ABSTRACT: Sliced male C57B1/10Sn (H-2-b) donor muscles were grafted into the female histocompatible muscles of untreated, FK506-treated, and T-cell depleted (with or without thymic tolerance) dystrophic (mdx; H-2-b) and normal (C57B1/10Sn; H-2-b) hosts, and also into histoincompatible normal (Balb/c; H-2-d) hosts. The fate of male donor nuclei was monitored on tissue sections by in situ hybridization with a Y-chromosome specific probe. The results demonstrate that the dystrophic environment is more conducive than normal muscle to donor myoblast migration, with the distance moved being threefold greater at 12 weeks in dystrophic hosts. T-cell depletion was significantly more effective than FK506 treatment at enhancing donor myoblast migration in both histocompatible and histoincompatible hosts at 3 weeks. Furthermore, the effects of T-cell depletion were sustained in histoincompatible hosts at 12 weeks. These data endorse the use of host T-cell depletion as a promising long-term strategy to improve myoblast transfer therapy (MTT) in the clinical situation.


ENHANCED MIGRATION AND FUSION OF DONOR MYOBLASTS IN DYSTROPHIC AND NORMAL HOST MUSCLE

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Duchenne muscular dystrophy (DMD) is an X-linked recessive disease in which a mutation in the dystrophin gene results in the absence of this protein from beneath the sarcolemma of most skeletal muscle fibers. Consequently, dystrophic muscle is characterized by a severe loss of sarcolemmal integrity, and the muscle progressively degenerates. Cell- and virus-mediated gene therapies for DMD have been widely explored. Transfer of the dystrophin gene into dystrophic muscle by injecting cultured myoblasts from normal donors (myoblast transfer therapy, or MTT) is considered to be one of the most promising candidates for the treatment of DMD. The success of MTT is dependent upon the survival of normal donor myoblasts within the dystrophic host environment, their migration away from the injection site, and their fusion with host myofibers. Early clinical trials with MTT showed limited success because few donor myoblasts survived within the host environment. Both clinical and experimental studies have demonstrated that the majority of cultured donor myoblasts die very rapidly after injection. This is due, at least in part, to an acute, nonspecific inflammatory response. Several studies have also demonstrated prolonged survival of a small proportion of injected cultured donor myoblasts in immunosuppressed or immunodeficient hosts. Beauchamp and colleagues showed that although most cultured donor myoblasts die within 1 h after injection, a small proportion survive and proliferate very rapidly in irradiated muscle of immunodeficient dystrophic host mice. These observations suggest that the early death of most injected cultured donor myoblasts may be compensated by the survival and proliferation of a small percentage of putative myogenic "stem cells" in the injected population. However, withdrawn or inadequate immunosuppression results in almost immediate and complete rejection of any surviving donor myoblasts, and the association of CD4+ and CD8+ T-cells with this phenomenon suggests that a specific, cell-mediated reaction is responsible. Therefore, the limited success of both experimental and clinical MTT trials

Abbreviations: DIG, digoxigenin; DMD, Duchenne muscular dystrophy; EDL, extensor digitorum longus; MHC, major histocompatibility complex; MTT, myoblast transfer therapy; TA, tibialis anterior

Key words: Duchenne muscular dystrophy; FK506; histocompatibility; myoblast transfer therapy; T-cell depletion

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Myoblast Migration and Fusion

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appears to be due to the combined effects of the incompatibility of cultured donor myoblasts with the host environment, a relatively early nonspecific inflammatory reaction, and a later, specific cell-mediated immune response. Many studies are addressing these aspects of survival of conditioned donor myoblasts after injection, but once this is achieved, it is imperative that these myoblasts can disperse within the host environment, and fuse with host myofibers. Unlike donor myoblast survival, very few studies have examined factors that might promote the migration and fusion of donor myoblasts into dystrophic host muscle.

Optimal donor myoblast migration is imperative to the eventual clinical application of MTT. In vitro studies using the Boyden chamber assay show that myoblasts can migrate in response to factors produced by damaged muscle, and to soluble chemotactic growth factors. Since many of these growth factors are produced at sites of skeletal muscle injury, it is likely that chemotactic signals produced by damaged muscle may also operate in vivo. In vitro evidence indicates that a main source of myoblast chemoattractants in injured skeletal muscle is macrophages, since (a) macrophages migrate towards sites of skeletal muscle damage, and (b) myoblasts migrate towards macrophage preparations. Attempts have been made at drawing parallels between these in vitro observations and myoblast migration in vivo. Myoblast migration in vivo is usually limited, but is significantly enhanced when the host muscle is injured or irradiated. The improved myoblast migration into injured muscle is consistent with the in vitro observations, and implicates a chemotactic environment for myoblasts at the site of injury. The three-dimensional extracellular matrix that supports all tissues in vivo provides a variable which is difficult to account for in vitro. Myoblasts can produce matrix-degrading enzymes in vitro, and the specific enzymes produced vary with changes in the culture substrate. Furthermore, the migration of myoblasts cultured with concanavalin A, which increases their expression of matrix-degrading proteases, is enhanced following their injection into uninjured host muscle. The observations provide strong support for the hypothesis that the fragmentation of the extracellular matrix caused by muscle injury or irradiation promotes myoblast migration compared to the intact matrix in uninjured skeletal muscle. Unfortunately, the use of irradiation and skeletal muscle injury are not clinically appropriate for enhancing myoblast migration in MTT, and so other methods are being explored. Since dystrophic muscle is characterized by foci of necrotic and regenerating myofibers, it seems likely that this alone might enhance donor myoblast migration. This possibility was tested in the present article by grafting sliced muscles from normal (C57B1/10Sn; H2-b) donor mice into the muscles of dystrophic (mdx; H2-b) hosts, and comparing the extent of donor myoblast migration with that seen in normal (C57B1/10Sn; H2-b) host muscle. The mdx mouse is a widely accepted dystrophic animal model for human DMD. Sliced muscle grafting is an established model of MTT in which donor myoblasts have been shown to survive for many months, and to readily migrate into and fuse with dystrophic host muscle. In this model, a Y-chromosome specific probe is used to track male donor myoblasts implanted into female hosts, using in situ hybridization on tissue sections. This technique allows the movement of male donor myoblasts away from sliced donor muscle grafts and into female host muscles to be visualized.

It has been established that the use of immunosuppressants such as FK506 diminish the specific immune response in MTT, enabling the survival of at least a proportion of injected cultured donor myoblasts. However, the effect of such immunosuppressant drugs on the migration and fusion of donor myoblasts has not been examined. Therefore, the present study also examines the effects of FK506 on the migration and fusion of donor myoblasts originating in normal sliced muscles grafted into FK506-treated dystrophic (mdx) hosts. Furthermore, the efficacy of FK506 for use in MTT across major histocompatibility complex (MHC) barriers was evaluated by grafting sliced muscles from normal donors (C57B1/10Sn; H2-b) into histoincompatible normal (Balb/c; H2-d) host mice treated with FK506. The effect of FK506 on donor myoblasts was examined at only 1 and 3 weeks, since the daily administration of this drug is expensive and tedious.

The survival, migration and fusion of donor myoblasts from sliced muscle grafts are significantly enhanced when peripheral T-cells of host mice are depleted, and naïve T-cells within the host thymus are tolerized to donor antigens. These results suggest that T-cell depletion and thymic tolerization may be an attractive alternative to the more rigorous, and often deleterious sustained long-term immunosuppressant treatments (such as FK506) that are commonly used clinically to promoted transplantation success. Hence, the data from T-cell depleted and tolerized host mice are compared with the data for FK506-treated mice.

The study by Fan et al. in 1997 examined sliced normal (C57B1/10Sn; H2-b) muscles grafted into
histocompatible dystrophic (mdx; H2-b) hosts for up to 12 weeks. These studies have now been extended to histoincompatible hosts. The final objective of the present study was to assess the effects of long-term (up to 12 weeks) T-cell depletion and thymic tolerization on the survival, migration, and fusion of male donor myoblasts from normal (C57B1/10Sn; H2-b) sliced muscles grafted into histoincompatible normal (Balb/c; H2-d) female host mice.

**MATERIALS AND METHODS**

**Animals.** A total of 27 female dystrophic (mdx; H2-b), 6 normal C57B1/10Sn (H2-b; the parent strain for mdx), and 15 normal Balb/c (H2-d) mice, aged 6-8 weeks, were used as the hosts. Forty-eight male normal (C57B1/10Sn; H2-b) mice, aged 8 weeks were used as the donors. All mice were obtained from the Animal Resources Centre (Murdoch University, Western Australia). Animals were housed in an environmentally-controlled room, and food and water were freely available. All animal procedures were undertaken in strict accordance with the guidelines of the National Health and Medical Research Council of Australia. The treatment protocols for each treatment group (A-K) are summarized in Table 1, and the flow diagram shown in Figure 1 demonstrates the comparisons made between these groups.

**FK506 Treatment.** FK506 was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). In the FK506-treated groups (Groups C and E), the drug administered daily to female host mice at an intraperitoneal dose of 5 mg/kg/day, beginning on the day of grafting and continuing throughout the duration of the experiment.

**T-Cell Depletion and Thymic Tolerization.** Host peripheral T-cell depletion and thymic tolerization were performed as described in detail by Fan et al. In brief, T-cell depletion was performed by intraperitoneal injection of monoclonal antibodies to the

![Figure 1](image_url)  
**FIGURE 1.** Flow chart demonstrating sliced muscle grafts from C57B1/10Sn male donors into different female host groups (as in Table 1), treatments of hosts, and the comparisons (+) made between groups for each experiment.

### Table 1. Summary of all treatment groups for all experiments.*

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*All sliced muscle grafts were from male donor C57B1/10Sn mice into female host mice of different strains.

*Numbers for muscle grafting refer to total number of hosts receiving sliced muscle grafts in both TA muscles.
CD4 and CD8 molecules (YTS191.1.2 and YTS169.4.2, respectively). Antibodies were administered on days 1, 3, and 7 prior to sliced muscle grafting on day 11. Tolerization of naive T-cells to donor antigens was induced by injecting cultured male donor (C57Bl/10Sn) myoblasts directly into the host thymus 7 days prior to grafting (day 4). T-cell depletion was administered either alone (Groups G and I), or in combination with thymic tolerization (Groups H and J).

Grafting. On the day of grafting (day 11), normal (C57Bl/10Sn) male donor mice were killed by halothane anesthesia. Both extensor digitorum longus (EDL) muscles were removed, sliced longitudinally to penetrate the epimysium, then grafted into longitudinal slits made in the midregion of the tibialis anterior (TA) muscles of female host mice. Grafts were made to both TA muscles of each host. At various times (1, 3, or 12 weeks) after grafting, the host TA muscles (containing the donor grafts) were removed, fixed in 4% paraformaldehyde, processed, and embedded in longitudinal orientation in paraform wax. A longitudinal section (5 μm) of each sample was stained with hematoxylin and eosin and assessed for the presence and morphology of the graft. Male donor nuclei were then localized in 5 μm sections of female host muscle tissue by in situ hybridization with the Y-chromosome specific, Y-1 probe.

Preparation of the Y-1 Probe. The plasmid containing the Y-1 insert was cloned, isolated, and purified from JM101 bacteria. The Y-1 DNA fragment was cut from the plasmid with the EcoRI restriction enzyme, extracted by gel electrophoresis, and purified from the gel band by electroelution, followed by phenol/chloroform extraction and ethanol precipitation. The purified DNA pellet was resuspended in sterile double-distilled water. The Y-1 yield was quantified by spectrophotometry, and the required amount labeled with digoxigenin (DIG) using the DIG-High Prime system (Boehringer Mannheim). The yield of labeled DNA was quantified by comparison on a dot blot with known quantities of control labeled DNA (DIG Nucleic Acid Detection Kit; Boehringer Mannheim). The specificity of the labeled Y-1 probe for male DNA was confirmed by slot blotting unlabelled denatured Y-1 fragment and control female DNA onto a nylon membrane (Hybond N+; Amersham), then hybridizing overnight with the denatured DIG-labeled probe at 65°C. Finally, the probe specificity for male DNA in situ was confirmed by hybridization of 5 μm sections of paraformaldehyde-fixed, paraffin-embedded male (positive) and female (negative) control tissues.

In Situ Hybridization. Sections (5 μm) of female host muscle containing male donor sliced muscle grafts were mounted on silanated glass slides. Prior to hybridization, sections were incubated in 0.2N HCl, predigested with proteinase K (25 μg/mL), then prehybridized in hybridization buffer (20 x SSC 0.01mM dextran sulphate + 0.5M deionized formamide). The probe was diluted in hybridization buffer and placed on the sections. The sections were coverslipped and temporarily sealed with rubber cement. Sections were placed in an oven at 100°C to cause DNA denaturation, snap-cooled on ice to prevent immediate DNA reconstitution, then hybridized overnight at 42°C. DIG was localized with an alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim), and visualized by color reaction with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP; Boehringer Mannheim).

Analysis. Two nonserial sections from each grafted host TA were analyzed under a light microscope fitted with an eyepiece graticule after Y-1 in situ hybridization. The graticule was 1 mm in length, with 0.01 mm graduations. It was positioned at 90° to the graft border. The total number of male donor (Y-1 positive) nuclei within the width of the graticule, and their individual distances from the graft border were determined. Where possible, the graticule was repositioned to include donor nuclei that were located up to 2 mm away from the graft. At least four nonoverlapping sample fields in each section were selected randomly as a representative sample of the grafted host muscle. The migration of donor cells across (rather than along) host myofibers was determined, and it was noted that the maximum possible distance from the graft to the outer edge of the host TA was about 2 mm. At each time point, 2–3 hosts (4–6 grafts) were sampled, so that at least 32 sample fields for each group, at each time point, were counted. The percentage of male donor muscle nuclei that were fused with (i.e., were lying within) female host myofibers was also determined. Fused male donor muscle nuclei were classified as such based on their central location within the myofiber (Fig. 2). The retention of central nucleation has been widely used to distinguish adult mouse myofibers which have undergone necrosis and regeneration.53,54,73

One-way analysis of variance (ANOVA) and the
general linear model were used to test the statistical significance of variation due to single and multiple variables respectively. In all graphs, the columns represent the mean value ± standard error of measurement.

**Controls.** In Experiment 1, a direct comparison was made between female dystrophic (mdx; Table 1, Group A) and normal (C57Bl/10Sn; Table 1, Group B) host mice receiving sliced muscle grafts from normal (C57Bl/10Sn) donor mice. No Y-positive myoblasts were detected in female muscle tissue containing no male sliced muscle graft, and so no control data are shown for Experiment 1. For Experiments 2–5, controls were age- and strain-matched female host mice receiving sliced muscle grafts from normal male (C57Bl/10Sn) donor mice (Table 1, Groups D, F, and K).

**Experiment 2: Effects of FK506 on Donor Myoblast Movement and Fusion in Histocompatible Dystrophic Hosts.** Five female dystrophic (mdx; Table 1, Group C) were treated with FK506, and received sliced muscle grafts from male normal (C57Bl/10Sn) donors. Muscles were sampled at 1 or 3 weeks after grafting, and analyzed by in situ hybridization with the Y-1 probe. Four control mdx hosts received sliced muscle grafts with no FK506 treatment (Table 1, Group D).

**Experiment 3: Effects of FK506 on Donor Myoblast Movement and Fusion in Histoincompatible Normal Hosts.** In order to examine the effects of FK506 on donor myoblast survival, movement, and fusion in histoincompatible host mice, 3 female normal (Balb/c; H2-d) hosts received FK506 treatment and sliced muscle grafts from male normal (C57Bl/10Sn; H2-b) donors (Table 1, Group E). Three control Balb/c host mice received sliced muscle grafts alone (Table 1, Group F). Host muscles were sampled 3 weeks after grafting and analyzed by in situ hybridization. The data for the movement and fusion of donor myoblasts in the treated and untreated Balb/c hosts were compared to those for the

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**FIGURE 2.** Light micrograph of female host muscle containing a sliced muscle graft from a normal male donor (graft not shown). Male donor myoblasts were localized by in situ hybridization with the Y-1 probe. Some male donor nuclei (filled arrows) are clearly lying in a central position (i.e., are fused) within female host myofibers which have presumably undergone regeneration. Other male donor nuclei (clear arrows) have not fused and are lying either in the myofiber periphery, or in the interstitium. Bar = 100 μm.
FK506-treated dystrophic hosts in Experiment 2 (Table 1, Group C).

**Experiment 4: Comparison of FK506 Treatment with T-Cell Depletion and Thymic Tolerization in Histocompatible Dystrophic Hosts.** Following either T-cell depletion (Table 1, Group G), or T-cell depletion plus thymic tolerization (Table 1, Group H), female dystrophic host mice (6 in each group) received sliced muscle grafts from male normal (C57Bl/10Sn) donors to both TA muscles. The dystrophic host mice receiving only sliced muscle grafts in Experiment 1 (Table 1, Groups A) were used as the controls. Host TA muscles were sampled at 3 or 12 weeks after grafting (3 hosts per group, per time point), and analyzed by in situ hybridization with the Y1 probe. To compare the effects of these treatment regimes with FK506, the 3-week data for the movement and fusion of donor nuclei from sliced muscle grafts were compared to those for the FK506-treated dystrophic hosts obtained in Experiment 2 (Table 1, Group C).

**Experiment 5: Long-Term Effects of T-Cell Depletion and Thymic Tolerization in Histoincompatible Normal Hosts.** Female normal (B6/Cg-H-2d) mice were T-cell depleted (Table 1, Group I), or T-cell depleted and thymic tolerized (Table 1, Group J), and received sliced muscle grafts from histoincompatible normal male (C57Bl/10Sn; H-2b) donors. Control hosts received sliced muscle grafts with no immunological therapy (Table 1, Group K). There were 3 hosts in each group, and all were sampled 12 weeks after grafting and analyzed by Y1 in situ hybridization to determine if these treatment regimes were effective at promoting donor myoblast survival, migration and fusion over a long period of time.

**RESULTS**

**Experiment 1.** The male C57Bl/10Sn sliced muscle grafts in histocompatible normal (C57Bl/10Sn) and dystrophic (mdx) female host mice were characterized by many large mature myofibers, most of which were centrally nucleated. Only occasional inflammatory cells were present in these grafts, and grain morphology did not change between 3 and 12 weeks after grafting. In addition, there were no obvious morphological changes in normal male sliced muscle grafts in histocompatible normal or dystrophic host mice treated with either FK506, or T-cell depletion/thymic tolerization.

In situ hybridization with the Y1 probe showed no significant differences in the total number of male donor nuclei found within the female host muscles (Fig. 3a), the distance that donor myoblasts were located from the graft (Fig. 3b), or the percentage that had fused with host myofibers (Fig. 3c), between dystrophic (mdx) and normal (C57Bl/10Sn) hosts at 3 weeks. However, at 12 weeks there were more male donor nuclei located within dystrophic host muscle tissue (Fig. 3a), and these were located further from the graft (Fig. 3b), compared to normal hosts. At 12 weeks, the maximum distance moved by any one male donor nucleus into dystrophic host muscle was 0.89 mm, which was almost threefold the maximum distance moved by one male donor nucleus (0.31 mm) into normal host muscle at this time point. These results indicate that the dystrophic environment is more conducive to the

![Figure 3](image)

**FIGURE 3.** Mean number (a), mean distance moved (b), and percentage fusion (c) of normal male donor nuclei migrating out from sliced muscle grafts and into the muscle of female dystrophic (mdx) and normal (C57Bl/10Sn) hosts at 3 and 12 weeks after grafting. The general linear model was used to determine if variations within and between groups were significant (*P < 0.05; **P < 0.005).
movement of donor cells out from sliced muscle grafts over a long period of time (12 weeks); however, in the short-term (3 weeks), it is no more influential than the normal muscle environment.

**Experiment 2.** The total numbers of male donor nuclei within female host muscles (Fig. 4a), their mean distance from the nearest graft border (Fig. 4b), and the percentage that had fused with host myofibers (Fig. 4c) were determined for FK506-treated and untreated (control) dystrophic (mdx) hosts. At 1 week after grafting, there was no difference in the number of male donor nuclei located outside the graft within host muscle, between treated and untreated hosts (Fig. 4a). However, by 3 weeks after grafting, the total number of male nuclei located within the host muscle tissue of FK506-treated hosts had doubled (P = 0.001), while no change over time was observed in the controls. Furthermore, the distance that donor nuclei were located from the graft was significantly higher at 3 weeks in hosts treated with FK506 compared to the untreated controls (Fig. 4b). This was the result of a threefold increase in the distance moved by donor nuclei in treated hosts between 1 and 3 weeks (P < 0.005).

At both 1 and 3 weeks after grafting, the percentage of male donor nuclei that had fused with (i.e., were lying within) host myofibers was significantly higher in the FK506-treated hosts compared to the untreated controls (Fig. 4c). No change in the fusion of donor nuclei in either group over time (between 1 and 3 weeks) was observed.

These results indicate that FK506 enhances the survival and/or migration of donor myoblasts out from sliced muscles grafted into dystrophic hosts by 3 weeks, and that their fusion with host myofibers is enhanced.

**Experiment 3.** Hematoxylin and eosin stained sections showed few obvious differences in the morphology of donor C57Bl/10Sn muscles grafted into FK506-treated and untreated histoincompatible Balb/c host mice at 1 week (data not shown). The grafts had a disorganized appearance and consisted of necrotic, and some calcified donor muscle tissue. They contained a few myotubes, although these were small and poorly formed compared to those in the adjacent regenerating host muscle that had been injured during surgery. The grafts were heavily infiltrated by inflammatory cells, although there were fewer in the adjacent host muscle in FK506-treated hosts. Analysis with the Y-1 probe showed some surviving myotubes of donor origin within the grafts in FK506-treated hosts, although they were surrounded by inflammatory cells, and the grafts were somewhat necrotic. Graft morphology at 3 weeks was similar, although fewer surviving male nuclei were present. Donor (male) nuclei were not detected in untreated control hosts at 1 or 3 weeks.

The ability of FK506 to promote the migration of donor myoblasts into muscles of histoincompatible hosts at 3 weeks was then examined. A mean of 17.4 male donor nuclei per sampling field was observed in the FK506-treated Balb/c hosts. The male nuclei had moved an average of 0.194 mm away from the graft, and 45% of them had fused with the host myofibers.

These data were compared to those for the FK506-treated histocompatible (dystrophic) mdx hosts at 3 weeks. Both the total number of male donor nuclei located within the host tissue (Fig. 5a), and the mean distance of these male nuclei from the...
graft (Fig. 5b) were significantly higher in the FK506-treated histocompatible mdx hosts compared to the histoincompatible Balb/c hosts. The maximum distance moved by any one male donor nucleus was 0.94 mm in mdx hosts, compared with only 0.57 mm in the Balb/c hosts. Furthermore, in the mdx hosts, there was a wider distribution of male donor nuclei (Fig. 5c). In FK506-treated dystrophic mdx hosts, 30% of the donor nuclei were located more than 0.5 mm away from the graft, compared to only 1.2% in the normal Balb/c hosts. The percentage of donor myoblasts that fused with host myofibers did not differ between the two types of FK506-treated hosts.

**Experiment 4.** Recent studies in our laboratory have shown that a short course of T-cell depleting antibodies, without or with an injection of donor myoblasts into the host thymus, enhances the survival, movement, and fusion of donor myoblasts from sliced muscle grafts into the muscle of dystrophic hosts. A reanalysis of the 3 week samples from this study and comparison of these data with the FK506-treated dystrophic hosts (Experiment 2 above) showed no significant differences in the number of male donor nuclei that had emigrated from the graft and into female dystrophic host muscle between the 3 treatment groups (Fig. 6a). All 3 treatment groups were superior to untreated control hosts. However, the distance that male donor nuclei were located from the graft was significantly lower in the FK506-treated hosts compared to the other treatment groups (Fig. 6b), although this was still much greater than in control hosts. Furthermore, the maximum distances moved by any one male nucleus for the FK506-treated, T-cell depleted, and T-cell depleted and tolerized hosts were 0.94 mm, 1.21 mm, and 1.15 mm.

**FIGURE 5.** Comparison of the effects of FK506 on (a) the total number of normal male donor nuclei from sliced muscle grafts located within host muscle, and (b) their mean distance away from the graft in histocompatible (mdx) vs. histocompatible (Balb/c) female hosts at 3 weeks. The general linear model was used to determine if variations due to treatment and host strain were significant (**P < 0.005**). The distance histogram (c) shows the distribution of male donor nuclei within female host muscle.

**FIGURE 6.** Comparison of the effects of FK506, T-cell depletion (A/B), and T-cell depletion with thymic tolerization (A/B + thy) on (a) the number of male donor nuclei that emigrate from sliced muscle grafts, (b) the mean distance they move away from the graft, and (c) their distribution within female dystrophic host muscle. Tissues were samples at 3 weeks after grafting. One-way ANOVA was used to compare the treatment groups with the controls (**P < 0.005**), and the treatment groups with one another (**P < 0.005**).
mm, respectively. T-cell depletion, with or without thymic tolerization, promoted a wider distribution of male donor nuclei within female dystrophic host muscle (Fig. 6c), compared with FK506 treatment. At 3 weeks after grafting, 22 and 18% of male donor nuclei were located further than 0.8 mm away from the graft in T-cell depleted, and T-cell depleted and tolerized hosts, respectively. This contrasted markedly with only 2.7% of donor nuclei that had moved further than 0.8 mm in the FK506-treated dystrophic hosts. No donor nuclei were found beyond 0.8 mm from the graft in the untreated control dystrophic hosts. This demonstrates that T-cell depletion, with or without thymic tolerization, is more effective than FK506 in facilitating the migration of donor myoblasts out from sliced muscle grafts and into dystrophic host muscles.

**Experiment 5.** The sliced C57B1/10Sn muscle grafts in T-cell depleted, and T-cell depleted and thymic Balb/c tolerized hosts were characterized by many fully developed, mature myofibers, and occasional clusters of inflammatory cells. When in situ hybridization with the Y-1 probe was used to localize the male donor nuclei, there was marked variation in the appearance of grafts between treatment groups. In the T-cell depleted, and T-cell depleted and tolerized Balb/c hosts, the sliced muscle grafts were characterized by numerous Y-1 positive nuclei, demonstrating the presence of cells of donor male origin. However, the graft and adjacent host muscle were devoid of donor nuclei in female Balb/c hosts that received only thymic tolerization with no T-cell depletion, and in control hosts receiving only a sliced muscle graft (data not shown). These results demonstrate that T-cell depletion, with or without thymic tolerization, promotes the survival and regeneration of sliced muscle grafts across MHC barriers, while thymic tolerization alone has no beneficial effect.

The migration and fusion of male C57B1/10Sn donor nuclei from sliced muscle grafts in the T-cell depleted, and T-cell depleted and thymic tolerized female Balb/c host mice were quantified and compared to the control mice receiving only a sliced muscle graft (since donor myoblasts did not survive in hosts treated with a thymic injection only, these tissues were not studied further). In the T-cell depleted Balb/c hosts, a mean of 19 male donor nuclei per field were observed within female muscles at 12 weeks after grafting (Fig. 7a). This was greater and highly significant compared to the T-cell depleted and tolerized, and the control (graft only) hosts ($P < 0.005$). However, this value was significantly lower than in T-cell depleted histocompatible dystrophic hosts at 12 weeks ($P = 0.002$; data not shown). The difference between numbers of donor (male) nuclei in the 2 treatment groups was also reflected in a significant difference in the mean distance moved in the 2 groups (Fig. 7b; compare A/B and A/B + thy).

Interestingly, there was no difference in the mean distance or the maximum distance that male donor nuclei were located from the graft in T-cell depleted histoincompatible normal (Balb/c) and histocompatible dystrophic (mdx) hosts at 12 weeks (Fig. 7b). This comparison was made between histocompatible dystrophic hosts (in which donor myoblast migration and fusion were maximal) and histoincompatible normal hosts to emphasize the beneficial effects of T-cell depletion across histocompatibility barriers. Like the dystrophic hosts, the maximum distance moved by any one male donor nucleus into Balb/c host muscle was 2 mm (which corresponded with the limiting border of the host TA). In T-cell depleted Balb/c hosts, 54% of the
male donor nuclei fused with female host myofibers at 12 weeks after grafting, which was significantly lower than the 78% that fused with dystrophic host myofibers \( (P = 0.017) \). In T-cell depleted Balb/c and mdx female host mice, the morphology of normal (C57B1/10Sn) male donor sliced muscle grafts was similar at 12 weeks after grafting (data not shown). In both cases, the grafts contained numerous densely packed, healthy myofibers with central, Y-1-positive nuclei, demonstrating excellent survival and regeneration of male donor sliced muscle grafts in histo-compatible dystrophic and histoincompatible normal female hosts treated with CD4+ and CD8+ T-cell depleting antibodies.

**DISCUSSION**

The results of this study demonstrate that the depletion of CD4+ and CD8+ T-cells in normal mice receiving histoincompatible sliced muscle grafts enables the survival of donor myoblasts within the graft, and promotes their emigration and fusion with host myofibers.

The survival, migration, and fusion of donor myoblasts are imperative to the success of MTT\(^a\). Many studies have successfully used immunosuppressants to promote donor myoblast survival.\(^{31,35,40,42,49,69,79}\) However, the migration and fusion of transplanted myoblasts has barely been addressed.\(^{16,46}\) The present study utilizes the sliced muscle graft model to specifically address the important issues of myoblast migration and fusion in dystrophic and normal hosts. In marked contrast with injected cultured donor myoblasts where the cells rapidly perish,\(^{17,19,22,30}\) myoblasts from sliced muscle grafts readily survive, migrate out from the graft into host tissue, and fuse with host myofibers.\(^{15}\) Furthermore, since the graft borders are clearly defined in tissue sections, the distance that donor cells have moved from the graft can be quantified.

**Donor Cell Migrate Preferentially in a Dystrophic Environment.** The movement of donor myoblasts within the normal and dystrophic muscle environment appeared the same at 3 weeks. However, at 12 weeks, donor cells had apparently migrated further into dystrophic muscle compared to normal (histo-compatible) muscle. Therefore, some aspect of the dystrophic environment favors the sustained migration of donor myoblasts, and this has not been previously demonstrated. It seems unlikely that differences in interstitial connective tissue can account for this disparity, since increased amounts of collagen occur in dystrophic muscle,\(^{31}\) and this would be predicted to impair myoblast movement.\(^{37,68}\) The simplest explanation is that over a long (12-week) period of time, the foci of degenerating/regenerating muscle and their associated chemotactic signals in dystrophic muscle may be important in the movement of myoblasts. If this is so, it implies that chemotaxis operates in vivo as well as in vitro.\(^4,20,62\) The dystrophic environment is likely to change during the disease, as the progressive loss of regenerative capacity of the muscle results in replacement by adipose and connective tissue. Such changes in structural integrity, and possibly in the soluble growth factors being produced by the dystrophic muscle, may have varied effects on the behavior of grafted normal donor myoblasts. Further investigation into the effects of the changing (aging) dystrophic environment on donor myoblast behavior may have clinical relevance. The results of the present study are encouraging for potential clinical MTT trials as they indicate that the dystrophic environment provides favorable conditions for the dispersion of injected cultured donor myoblasts away from the injection site. It is recognized that, while being striking in the mouse model, the migratory distances observed so far (less than 2 mm) are of limited significance with respect to the treatment of human dystrophinopathies at this stage. Long-term studies in larger models are essential to clarify the extent to which eventual myoblast movement might occur.

Another factor that influenced the emigration of donor myoblasts was the immune status. In normal host mice, the emigration of donor myoblasts was greater in histoincompatible Balb/c hosts (treated with T-cell depleting antibodies) compared with histo-compatible C57B1/10Sn hosts. The presence of inflammatory cells around such incompatible grafts (even at 12 weeks; Fig. 6) suggests that these cells might modify the interstitial spaces of the host muscle to facilitate myoblast movement. Another strong possibility (that we favor) is that the antibody treatment (used only in Balb/c hosts) was responsible for this difference in myoblast movement between these two strains of normal mice, since such antibody treatment clearly has this effect in dystrophic (mdx) muscle.

In contrast to myoblast migration, the fusion of donor myoblasts with host myofibers did not appear to be enhanced in the dystrophic environment at either 3 or 12 weeks after grafting. This was surprising, because the ongoing regenerative process in dystrophic muscle of mdx mice was expected to provide more opportunities for donor myoblast fusion. Because cell types other than myoblasts (e.g., fibroblasts) may also be migrating out of the sliced muscle grafts into host muscle, it is possible that a higher
percentage of the cells migrating out from the sliced muscle grafts are fibroblasts in the dystrophic environment and, since these male fibroblasts would not fuse, this could account for the lack of increase in the proportion of donor cells that fuse with host myofibers. An alternative theoretical possibility is that an increased number of myoblasts emigrate from the grafts in the dystrophic environment but their ability to fuse is impaired. Yet another possibility is that the donor myoblasts are subsequently lost from mosaic myofibers due to subsequent necrosis of the newly formed myofibers. It has recently been demonstrated that one cycle of necrosis and regeneration of dystrophic myofibers does not necessarily protect these myofibers from recurrent necrosis (although this study did not examine the effects of MTT on host myofiber necrosis). Since such subsequent myofiber breakdown can occur within 10 days of the initial repair event in dystrophic muscle, the instability of the regenerating mdx myofibers (even where normal myonuclei may be incorporated) provides ample opportunity for the demise of such fused male donor nuclei.

That dystrophic (mdx) myofibers which contain normal donor myoblasts following MTT can be protected from subsequent necrosis was demonstrated by Morgan et al. The critical question is “how many normal donor nuclei are required to prevent necrosis?” In terms of MTT, the percentage of normal donor nuclei that fuse with dystrophic myofibers is likely to reflect the amount of dystrophin expressed by these mosaic fibers. Karpati et al. and Chamberlain calculated that restoration of 10–20% of normal dystrophin expression by most myofibers is required to correct the muscle pathology. More recently it has been reported that at least 40% of host myofibers must express dystrophin for MTT to be deemed successful. These discrepancies may be due to different assessment techniques; as in the first two studies, expression levels within individual myofibers were assessed, whereas in the third, the overall number of dystrophin-positive fibers was determined. In the present study, it is assumed that the fusion of normal (dystrophin-positive) donor myoblasts with dystrophic (dystrophin-negative) host myofibers imparts the ability to synthesize and express dystrophin. Current studies in our laboratory are investigating the extent of dystrophin expression, and its correlation with numbers of donor male nuclei within female host myofibers.

**FK506 Promotes Donor Cell Migration and Fusion.** FK506 has been used clinically for organ transplantation and experimentally for cell and adenovirus-mediated gene transfer. FK506 also prevents the rejection of injected donor myoblasts in monkeys and dystrophic mdx mice, and significantly enhances the expression of dystrophin in mdx mice. The FK506 promotes the migration of donor myoblasts into the muscle of dystrophic hosts is demonstrated by the present study. Furthermore, a significantly higher percentage of these cells, presumably myoblasts, fused with host myofibers in FK506-treated hosts compared to the untreated control hosts. These results strongly support the use of FK506 in MTT, not only to prevent the death of transplanted donor myoblasts, but also to promote their migration away from the graft site and their fusion with host myofibers. Moreover, the finding that FK506 also promoted the survival, movement, and fusion of donor cells from sliced muscle grafts in histoincompatible hosts (albeit far less than in histocompatible hosts) indicates that FK506 is potentially useful for cell transplantation across major histocompatibility barriers.

**Evaluation of Different Immunosuppressive Strategies.** FK506 appears to have multiple modes of action. It inhibits interleukin-2 production, thereby preventing the proliferation and differentiation of CD4+ and CD8+ T-cells. It also downregulates natural killer cell number and activity, and the expression of endothelial cell adhesion molecules which mediate leukocyte extravasation. However, while being an effective immunosuppressant, FK506 has been reported to have some deleterious side effects, such as nephro- and neuro-toxicity. Cyclosporine is another immunosuppressant widely used clinically, and of particular concern is the recent report that this drug promotes malignancy via transforming growth factor-β upregulation. A major disadvantage of immunosuppressants such as FK506 and cyclosporine is that they are required on a long-term basis in MTT, as their withdrawal results in the death of injected donor myoblasts. Overall, it has been the general opinion that the immunosuppressive capabilities of these drugs far outweigh their adversity.

Because of the undesirable side effects of long-term immunosuppressants, a previous study in our laboratory examined the use of monoclonal antibodies to ablate specific T-cell subsets as an alternative immunosuppressant strategy in MTT. This showed that the depletion of circulating CD4+ and CD8+ T-cells, with or without tolerizing naive T-cells to donor antigens, enhances the survival, migration, and fusion of donor myoblasts from sliced muscles.
grafted into dystrophic hosts. These treatment regimens were effective for at least 12 weeks after grafting, and were only required as a single short course prior to transplantation. These results support the use of T-cell depletion and thymic tolerization as long-term immunosuppressive strategies in MTT. However, the efficacy of these treatment regimes has not been compared to the more commonly used FK506. In the present study, it was demonstrated that over the 3-week period following muscle grafting, T-cell depletion and thymic tolerization had a highly significant beneficial effect on the distance that donor cells moved away from the graft compared to FK506. The data show that T-cell depletion and thymic tolerization is not only as effective as FK506 at promoting the survival of donor cells originating in sliced muscle grafts, but that the former more strongly enhances the migration of these cells away from the graft site. In addition, this treatment regime is required only on a short-term basis and has no reported side effects.\textsuperscript{16} Studies are in progress in our laboratory to determine the long-term effects (up to 1 year) of these treatment regimes in MTT.

Treatment with both FK506 and T-cell depleting antibodies resulted in enhanced migration of normal male donor myoblasts from sliced muscle grafts into histocompatible normal and dystrophic, and histoincompatible normal female host muscle. The mechanisms by which this occurs are not clear, although there are several possibilities. A likely explanation is that enhanced donor myoblast migration is simply a reflection of their enhanced survival, resulting in a larger population of donor cells available to enter the host muscle. However, the possibility that these treatment regimes have an effect on migration independent of survival cannot be excluded. Wernig and Irintchev\textsuperscript{18} demonstrated that cyclosporine treatment induces a skeletal muscle damage, and since myoblast migration is enhanced in injured or irradiated muscle,\textsuperscript{32,42,50,53,54,75,76} the results of the present study may be due to a similar effect on host muscle of FK506 and T-cell depletion. It must also be considered that T-cells inhibit donor myoblast migration, and that the inactivation or absence of T-cells (induced by FK506 or T-cell depleting antibodies, respectively) results in enhanced donor myoblast migration. Such an effect may be mediated by T-cell derived cytokines that inhibit myoblast chemotaxis either directly, or indirectly by downregulating myoblast chemoattractants from other sources.\textsuperscript{20,82,71}

The maximum possible distance that donor myoblasts could migrate in this model is restricted to about 2 mm. This distance was achieved by donor myoblasts at 12 weeks in both histocompatible dystrophic, and histoincompatible normal hosts after T-cell depletion. These results are based on the observation that this distance was achieved by a single donor myonucleus; the observation that individual donor myoblasts may have migrated this maximum possible distance in other treatment groups, or at earlier time points, cannot be excluded. However, T-cell depletion also had the most potent effect on the mean migratory distance, and the overall distribution of donor myoblasts, strengthening the conclusion that this treatment regime may be beneficial in MTT. The results of the present study have demonstrated that donor myoblasts from sliced muscle grafts have the potential to attain maximal migration, forming the basis for ongoing studies in our laboratory that are investigating the mechanisms by which donor myoblast migration is controlled, influenced, and possibly further enhanced.

In order to further validate T-cell depletion and thymic tolerization as a potential immunosuppressive treatment regime for MTT, the efficacy for muscle grafts transplanted into histoincompatible hosts was examined at 12 weeks. The depletion of host CD4+ and CD8+ T-cells enhanced the survival of these incompatible grafts which would normally be rapidly rejected (within 1 week). In an early study, Cobbold et al.\textsuperscript{11} demonstrated that the YTS191.1.2 and YTS169.4.4 monoclonal antibodies depleted CD4+ and CD8+ T-cells at efficiencies of 86% and 96%, respectively. This group later showed that skin allotransplant survival could only be prolonged beyond 70 days in histoincompatible hosts treated with these antibodies in combination with T-cell blocking (non-depleting) antibodies.\textsuperscript{12} These results suggest that a complete (100%) blockade of T-cells would be required to facilitate long-term survival of skin allografted across MHC barriers. Skin allografts are characteristically "a tough test of any tolerogenic protocol."\textsuperscript{74} The present study is one of the first to address the effects of the same T-cell depleting antibodies on muscle allograft survival in histoincompatible hosts, and the results are very promising, demonstrating prolonged survival for up to 84 days. Long-term studies (beyond 84 days), the effects of combined T-cell depleting and blockade, and an examination of the effects of T-cell depletion on the survival of a second-set muscle graft are essential to determine the extended feasibility of such potential muscle allografting across histocompatibility barriers.

Enhanced movement and fusion of donor myoblasts within incompatible host muscle was also seen in the present study. In marked contrast, very few donor cells were observed within the host muscle of
mice where thymic tolerization was carried out in addition to T-cell depletion. In this situation, not only did thymic injection fail to enhance the survival of myoblasts from histoincompatible sliced muscle allografts, but it appeared to annul the effects of peripheral T-cell depletion. These results contrast with other findings where intrathymic injection of donor antigens has been shown to confer donor-specific unresponsiveness on histoincompatible hosts.\(^5\)\(^6\)\(^8\) However, Alfrey et al.\(^8\) showed that the response of thymic tolerized host rats to heart allografts can vary depending on the donor strain. The failure of cultured donor myoblasts injected into the host thymus to induce tolerance to donor muscle grafts in the present study may, therefore, be due to the effects of the particular strain combination used; although the mechanisms by which thymic injection abrogated the effects of peripheral T-cell depletion are unclear. Since it has been demonstrated that the culturing process can alter the expression of neoantigens by donor myoblasts,\(^9\) the injection of cultured donor myoblasts into the host thymus may induce tolerance to different antigens to those present in the (uncultured) sliced muscle grafts.

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