# Increased Survival, Movement and Fusion of Myoblasts from Sliced Muscle Grafts into Skeletal Muscles of T-Cell Depleted and Tolerised Dystrophic Host Mice

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**Abstract**

A potential possible therapy, Myoblast Transfer Therapy (MTT), for the lethal childhood myopathy Duchenne Muscular Dystrophy (DMD), is the transplantation of myogenic cells (myoblasts) into the diseased skeletal muscle in order to replace the missing gene product, dystrophin. To date, experimental studies in animal models and clinical trials of this therapy in patients have shown little or no success. Immunological rejection of the transplanted (donor) myoblasts appears to be one of the major problems. To overcome this problem, the effect of immunomanipulating the host environment was investigated using female dystrophic *mdx* mice and a Y-chromosome specific probe to track the donor male myoblasts. Peripheral T-cells of adult female *mdx* host mice were depleted using anti-CD4 and CD8 antibodies, and naive T-cells were tolerised to the donor male myoblasts (C57Bl/10Sn) by injection of these myoblasts "directly into the host thymus. Grafts of sliced donor male muscle were then implanted into leg muscles of these treated *mdx* hosts and the muscles sampled after 1, 3 and 12 weeks. The migration of donor myoblasts away from the sliced muscle grafts and their fusion with host myofibres was significantly enhanced in muscles of the immunomanipulated hosts, compared with untreated hosts. This was marked at 3 weeks and sustained at 12 weeks. Thus, depletion of peripheral T-cells either alone or combined with induction of host tolerance, significantly enhances the success of MTT and represents a promising long-term strategy without the adverse side effects associated with long-term use of immunosupressive drugs.

**Key words:** myoblast transplantation, muscle graft, T-cell depletion, T-cell tolerance.

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Myoblast Transfer Therapy (MTT) is a potential treatment for the lethal myopathy, Duchenne Muscular Dystrophy (DMD). The principle of the therapy is to introduce the missing gene into dystrophic muscles by fusion of normal myoblasts with affected myofibres. Some success has been achieved in animal models, however, clinical trials undertaken in USA, Canada and Italy have met with minimal success [14, 17, 18, 22, 28, 31, 44, 45]. More information is required at the cellular and molecular level in order to understand the problems and to enhance the success of this therapy.

In myoblast transplantation studies there is rapid death (within 1 week) of cultured donor myoblasts after injection into 'untreated' host muscles [6, 11, 18]. The superior survival of similar donor myoblasts in immunosuppressed or immunocompromised host mice [19, 24, 25, 34, 46, 47] indicates that the failure of myoblast survival in 'untreated' hosts may, in part, be due to immunological factors. It had already been demonstrated that changes to the immunological status of the host can affect the survival and fusion of transplanted myoblasts [49].

In contrast, when whole or sliced muscle grafts (instead of cultured myoblasts) were implanted into muscles of "untreated" hosts they showed excellent survival for up to one year [12]. Furthermore, with sliced muscle grafts, emigration of donor cells into the host muscles was observed and many donor myoblasts fused with host muscle fibres. The present studies with sliced muscle grafts were designed to further enhance the movement and fusion of such transplanted donor myoblasts, based on evidence that immunological factors appear to play a role in this process [11, 12, 34, 35].

Immunosuppressive drugs, such as cyclosporine A (CsA) and FK506 have been used in MTT studies. The

success of MTT in mdx mice with CsA immunosuppression has been limited [20, 26, 27, 41, 50]. FK506, a macrolide, has strong immunosupressive activity and has been reported to be more potent than CsA *in vitro* [23] and *in vivo* [15, 25]. FK506 has been used successfully in children for heart transplantation [2] but side effects have been reported (drowsiness, anorexia, weight loss, lymphopenia, renal dysfunction) in recipient animals and humans [33, 42]. The well recognised adverse side effects associated with sustained use of immunosuppressive drugs are a major problem. Furthermore, some immunosuppressive agents may be directly toxic to host muscle [29]. An alternative strategy is to 're-educate' the host immune system to become tolerant to donor cells of interest. Such induction of tolerance is relatively easy to achieve in the immature system of the neonate [7] and recently this has been demonstrated in adult animals.

To tolerise adults to strong alloantigens, it is necessary to inhibit the ability of the peripheral T-cells to mount a rejection response. This has been achieved by the introduction of antibodies to specifically ablate T-cells *in vivo* [8, 9]. It has been shown that antibody depletion of $CD4^+$ and $CD8^+$ T-lymphocytes in the adult resulted in transplantation tolerance which was effective across multiple minor histocompatibility and MHC class I antigen combinations [36, 39, 48]. Antibodies to mouse CD4 (YTS 191.1.2) and CD8 T-lymphocytes (YTS 169.4.2.) have been used to achieve specific tolerance to MHC-mismatched skin without the need for any other source of tolerogen [10]. Similar experiments in rats have shown that depletion of host T-cells with specific antibodies can overcome the ability of peripheral T-cells to mount rejection responses and thus achieve successful cell transplantation [38]. The production of long-term host tolerance to implanted donor myoblasts using antibodies to deplete T-cells of adult hosts could obviate the need for long-term drug immunosuppression and its unfortunate side effects in MTT studies.

An additional approach to tolerance induction is the inoculation of donor antigen directly into the host thymus in a pretransplant strategy [37]. T-cells mature in the thymus and it is here that each set of T-cells is "educated". This process involves inactivation or deletion of auto-reactive T-cell clones and positive selection for clones capable of recognising foreign antigens [21, 32]. When the developing T-cells mature in a thymus containing a foreign tissue (donor antigen) then those T-cells will recognise the donor tissue as "self"and therefore not treat it as foreign. By transplanting donor cells, as the antigen, into the thymus of MHC incompatible rats, Posselt *et al* [37] appear to have persuaded the recipient host immune system to accept the donor transplant as "self rather than as a foreign invader. In these experiments the grafted tissue survived in the thymus permanently, after a brief period of immunosuppression. Combining the depletion of host peripheral T-cells (by using specific antibodies), with induction of tolerance in immature T-cells in the host thymus (by introducing donor antigens), could provide an effective long-term strategy to overcome the immunological problems related to MTT. Experiments to test this approach were carried out using the *mdx* mouse model of DMD and a Y-chromosome specific probe [13] to track normal male donor myoblasts transplanted into muscles of female *mdx* host mice [11, 12].

## Materials and Methods

*Mdx* and C57Bl/10Sn (the normal parental strain for *mdx)* mice were obtained from the Australian Neuromuscular Research Institute and the Animal Resource Centre, Western Australia. All animal procedures were carried out in strict accordance with guidelines of the National Health and Medical Research Council of Australia.

### *Depletion of host T-cells with antibodies*

T-cell depletion was performed by intraperitoneal injection of mAb to CD4 (YTS191.1.2.) and CD8 (YTS 169.4.2.) at a dose of 0.5-1.0 mg each in a total volume of 0.2 ml per mouse. Injections were given on three occasions; 3 days before, 1 day before and 3 days after myoblasts injection into the thymus of the mouse (Table 1). Muscle grafts were implanted into TA muscles 4 days after the last mAb injection. A total of 13 female *mdx* mice were injected with the antibodies. Effectiveness of the T-cell depletion was assessed by flow cytometry (FACScan, Becton Dickinson) on spleen cells derived from representative mice at the time of muscle engraftment.

### *Induction of tolerance to developing T-cells in the host thymus*

Skeletal muscles of male C57Bl/10Sn mice aged less than 6 weeks were taken from the hind limbs and lower back. Myoblasts were isolated from these muscles by enzyme digestion and filtration through 100 μm nylon gauze as described by Maley *et al* [30]. The cells were cultured for 3 days in Dulbecco's Modified Minimal Essential Medium (ICN-Flow) with 10% horse serum (Cytosystems, Australia) and harvested by digestion with 0.1% trypsin (ICN-Flow). The concentration of the cell suspension was adjusted to 1-5 x $10^7$ cells/ml with phosphate buffered saline pH 7.2 prior to injection. The myoblast suspension was injected into host thymi using a Hamilton syringe with a 26G needle. The needle was inserted vertically into the thymus through the chest skin with the mouse lying on its back. Two injections were made into each lobe of the thymus to give a total of four injections per thymus. A total volume of 20 μl of cell suspension was injected into each thymus. To check the effectiveness of thymic injection, Omnipaque (Sanofi Winthrop, Australia) was injected into the thymi of 4 mice and observed under CT scan (Toshiba, Japan). This confirmed the accuracy of the thymic injections (data not shown). Injected thymi were sampled at 1, 3 and 12 weeks and sections were analysed with the Y-1 probe.

Preliminary studies in our laboratory (unpublished observations) showed that thymic injection alone (without

*Table 1. Regime for T-cell depletion experiments using sliced male C57Bl/10Sn EDL muscles grafted into TA muscles of female* mdx *mice.*

| TIME | TREATMENTS | HOSTGROUPS A x5 | B x5 | C x4 | D x2 | E xl | F x1 |
|---|---|---|---|---|---|---|---|
| Day 1 | Antibody injection | ✓ | ✓ | | ✓ | ✓ | |
| Day 3 | Antibody injection | ✓ | ✓ | | ✓ | ✓ | |
| Day 4 | Mpc injection: thymus | | ✓ | | | ✓ | |
| Day 7 | Antibody injection | ✓ | ✓ | | ✓ | ✓ | |
| Day 11 | Muscle grafting: both TAs | ✓ | ✓ | ✓ | | | |
| | Sampling | | | | ✓2* | ✓1* | ✓1* |
| Day 18 | Sampling (1 week) | ✓2 | ✓2 | ✓2 | | | |
| Day 32 | Sampling (3 weeks) | ✓3 | ✓3 | ✓2 | | | |
| Day 95 | Sampling (12 weeks) | ✓3 | ✓2 | ✓2 | | | |

Note: Numbers in the table are the numbers of the host mice in the group. * Spleens were taken for analysis of T-cells.

antibody depletion of T-cells in the periphery) was not effective and therefore this regime was not tested in the present study.

*Muscle grafting and tissue analysis*

Male C57Bl/10Sn donor mice aged up to 8 weeks were used. The EDL muscles were gently separated, removed and sliced into three sections (longitudinally or transversely) as shown in Fig. 1. The sliced male donor muscle was inserted into female host TA muscle into a slit cut either longitudinally (Fig.1A) or transversely (Fig.lB). The host TA muscles with the grafts (and thymi) were sampled at 1, 3 or 12 weeks after transplantation (Table 1). Tissues were fixed, processed through paraffin and 5 μm sections hybridised with digoxigenin-labelled Y-1 probe *in situ* as described [11, 12, 13]. The photomicrographs were taken using Leitz light microscopy with Kodak film.

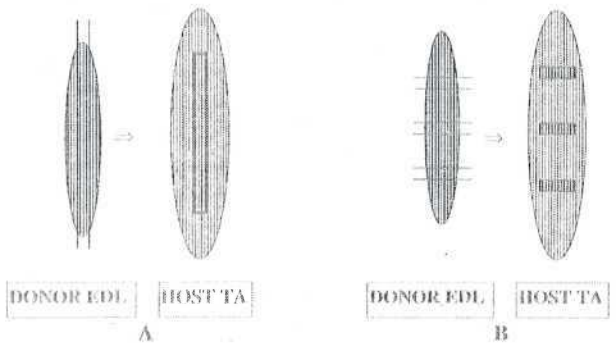


*Figure 1. Diagram of sliced male C57Bl/10Sn EDL muscle grafted into female mdx TA muscle. A: longitudinally sliced EDL graft placed into host TA muscle longitudinally. B: transversely sliced EDL graft placed into TA muscle transversely.*

*Experimental summary (Table 1)*

To deplete the peripheral T-cells of the female hosts, the antibody treatment, with YTS 191.1.2. and YTS 169.4.2., was administered at day 1, day 3 and day 7 of the experiments. At day 4, primary myoblasts from muscles of male donors (which were also used as a source of implanted donor muscle grafts in MTT), were injected into the thymi of some female host mice to tolerise the developing T-cells. Sliced muscle grafts of male C57Bl/10Sn mice were then transplanted into the TA muscles of these immunomanipulated host mice at day 11. Female host mice without any treatment but transplanted with sliced muscles were used as controls. The TA muscles and thymi of the hosts were taken at 1, 3 or 12 weeks after the grafting for *in situ* hybridisation analysis with the digoxigenin-labelled Y-l probe. Spleens of mice which were untreated, antibody treated, and antibody treated and injected with donor cells into thymus were taken for $CD4^+$ and $CD8^+$ cell analysis by flow cytometry at the day when muscle grafting was performed.

**Results**

*Effectiveness of host T-cell depletion and tolerance*

The spleens of antibody-treated (Table 1, Group D), antibody treated and thymus injected (Table 1, Group E), or untreated control (Table 1, Group F) host mice were sampled at 11 days after the start of the antibody injections at the time when the donor muscles were grafted into host mice. The numbers of $CD4^+$ and $CD8^+$ cells were examined by flow cytometry. It was found that in spleens of the hosts where antibodies had been administrated, both $CD4^+$ and $CD8^+$ cells were almost completely absent regardless of whether or not donor cells had also been injected into the thymus (Fig. 2). Thus the T-cell depletion by the antibodies appeared to have been effective.

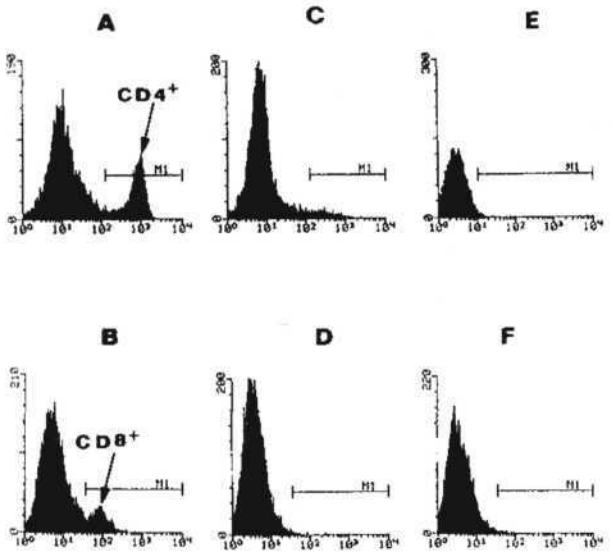


*Figure 2. Flow cytometry analysis showing the distribution of $CD4^+$ and $CD8^+$ cells in spleens of mdx mice either untreated (A, B), treated only with the antibodies against $CD4^+$ and $CD8^+$ (C, D), or treated with the antibodies and injected with C57Bl/10Sn myoblasts into the thymus (E, F). The $CD4^+$ (A, C, E) and $CD8^+$ (B, D, F) cells were present in the spleens of normal mdx mice (see peaks shown by arrows, on A and B respectively) but absent from the spleens of treated mdx mice.*

Sections of all injected and some uninjected thymi were analysed with the Y-1 probe. Many Y-1 probe positive male nuclei were present in the sections of all injected thymi (Fig. 3) but, as expected, hybridisation was not seen to any sections of uninjected female thymi. This confirms the success of the thymic injection and demonstrates long-term survival of the injected myoblasts within the thymi.

*Survival, movement and fusion of male donor cells from sliced C57Bl/10Sn muscle grafts in T-cell depleted and tolerised female* mdx *host mice*

The object of this experiment was to compare the survival (Table 2) and movement (Tables 2 and 3) of donor myoblasts from sliced muscle grafts, in T-cell depleted hosts, T-cell depleted and tolerised hosts, with untreated hosts, and the fusion of the donor cells with host muscle fibres in these three groups. The movement and fusion of donor cells from male C57Bl/10Sn sliced muscle grafts into female *mdx* mice were examined with digoxigenin-labelled Y-1 probe *in situ*. The grafts were successfully located in all hosts, except for the right legs of mice #1 and #11 and the left leg of mouse #5.

One week

One week after grafting, many donor (male) cells had moved out of the grafts into the host muscles and some of the male nuclei were within the host muscle fibres (Fig. 4). In 2 TA muscles of the antibody-treated hosts (mice #1 and #2), 314 donor cells were found, with 162 of them within

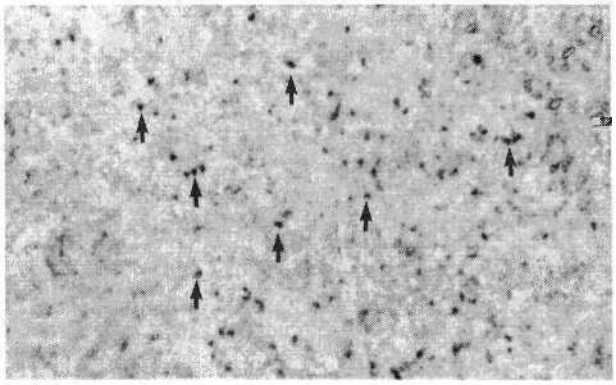

*Figure 3. Y-1 probe* in situ *analysis of female thymus injected with donor male myoblasts at day 4 and sampled at 3 weeks. This typical section shows many Y-1 positive nuclei (some examples shown by arrows) throughout at the female thymus. Magnification is x400.*

and 108 between host muscle fibres. The maximal distance moved by these male donor cells from the graft site was 1.1 mm. In 4 TA muscles of the hosts (mice #9 and #10) pretreated with antibodies and thymus injection, 412 donor cells were found, with 191 of them within and 149 between host muscle fibres. The maximal distance moved by these cells was 1.1 mm. In 4 TA muscles of untreated hosts (mice #16 and #17), 224 donor cells were found, with 144 of them within and 58 between host muscle fibres. The maximal distance moved by these cells was 1.1 mm.

More donor cells had moved out of grafts in the antibody and antibody/thymus treated hosts compared with the untreated hosts (average numbers of donor cells/sample were 157, 103 and 56 respectively, $P < 0.025$). However, the distance moved by the cells was similar in all 3 groups at 1 week ($P > 0.05$).

There was no striking difference between results for grafts made into a longitudinal or transverse slit in the host muscle ($P > 0.05$).

Three weeks

Three weeks after grafting, in 4 TA muscles of antibody-treated hosts (mice #3, #4 and #5), 453 donor cells were found, with 266 of them within and 158 between host muscle fibres (Fig. 4A). The maximal distance moved by these cells was 2.2 mm. In 5 TA muscles of hosts (mice #11, #12 and #13), pre-treated with antibodies and thymus injection, 536 donor cells were found, with 284 of them within and 169 between, host muscle fibres. The maximal distance moved by these cells was 2.3 mm. In 4 TA muscles of untreated hosts (mice #18 and #19), 88 donor cells were found, with 62 of them within and 17 between host muscle fibres (Fig. 4B). The maximal distance moved by these cells was 1.0 mm. In all 3 cases, the donor nuclei which had moved the maximal distance were located within muscle fibres.

In untreated hosts, there was a more than 50% decrease in the number of surviving migrated donor cells between 1 and 3 weeks (average numbers of migrated cells were 56

*Table 2. Number and location ofemigrated male nuclei derived from sliced muscle grafts ofmale C57Bl/10Sn mice inserted into TA muscles of (A) antibody-treated, (B) antibody/thymus-treatedor (C) untreated female* mdx *hosts: analysis with Y-J probe in situ.*

| Mouse # | Sampling Time | Number and Location of Y-Positive Cells (Male) in Female Host Muscles | | | | | |
|---|---|---|---|---|---|---|---|
| | | Left TA (Transverse Slit) | | | Right TA (Longitudinal Slit) | | |
| | | within MF | between MF | either | within MF | between MF | either |
| | | | | A. Antibody-treated hosts | | | |
| 1 | 1 week | 84 | 56 | 25 | * | * | * |
| 2 | 1 week | 78 | 52 | 19 | ** | ** | ** |
| 3 | 3 weeks | 55 | 31 | 8 | *** | *** | *** |
| 4 | 3 weeks | 96 | 66 | 10 | 72 | 38 | 4 |
| 5 | 3 weeks | * | * | * | 43 | 23 | 7 |
| 6 | 12 weeks | 8S | 40 | 21 | 62 | 33 | 20 |
| 7 | 12 weeks | 64 | 28 | 18 | 59 | 43 | 13 |
| 8 | 12 weeks | 91 | 67 | 5 | 77 | 27 | 6 |
| | | | | B. Antibody/thymus-treated hosts | | | |
| 9 | 1 week | 67 | 51 | 13 | 47 | 38 | 22 |
| 10 | 1 week | 48 | 33 | 20 | 29 | 27 | 17 |
| 11 | 3 weeks | 63 | 25 | 12 | * | * | * |
| **12** | 3 weeks | 77 | 37 | 14 | 57 | 34 | 16 |
| 13 | 3 weeks | 53 | 46 | 22 | 34 | 27 | 19 |
| 14 | 12 weeks | 9S | 44 | 32 | 68 | 41 | 12 |
| 15 | 12 weeks | ** | ** | ** | 89 | 21 | 44 |
| | | | | C. Untreated (control) hosts | | | |
| **16** | 1 week | 49 | 25 | 6 | 22 | 8 | 9 |
| 17 | 1 week | 35 | 4 | 4 | 38 | 21 | 3 |
| 18 | 3 weeks | 19 | 7 | 2 | 18 | 5 | 1 |
| 19 | 3 weeks | 8 | 3 | 4 | 17 | 2 | 2 |
| 20 | 12 weeks | 18 | 3 | 5 | ** | ** | ** |
| 21 | 12 weeks | 14 | 6 | 5 | 13 | 4 | 3 |

Note: Numbers in the table are the average number of the Y-1 positive cells on each section; MF: myofibre(s); * Graft was not found; ** Graft was found on surface of TA muscle; *** Y-probe hybridisation positive data, but not clear with respect to graft position. ' These positive cells were closely associated with the myofibres and whether they were located within or without could not be determined with certainty.

and 22 respectively, $P < 0.025$). Data from an earlier study [12] also showed a 50% reduction in the number of surviving donor cells within muscles of untreated hosts over time (average of 73 and 30 cells at 1 and 3 weeks respectively, $P < 0.025$). This decrease over time was not seen in the antibody and antibody/thymus treated hosts: (average numbers of migrated donor cells for the antibody and antibody/thymus treated hosts were 113 and 107 respectively at 3 weeks, $P > 0.05$).

The distance moved by the donor cells in the antibody and antibody/thymus treated hosts at 3 weeks was greater than at I week after grafting (Table 3). The average distance moved by the donor cells for the antibody and antibody/thymus treated hosts at 1 week was 0.59 mm and 0.56 nun respectively, compared with 0.91 mm and 1.18mm ($P < 0.0005$) at 3 weeks. This difference was not seen in the untreated hosts (average distance moved at 1 week and 3 weeks was 0.68 mm and 0.66 mm respectively, $P > 0.05$). There was also a greater difference in the distance moved by the donor cells at 3 weeks after grafting between antibody and untreated ($P < 0.0005$), and antibody/thymus treated and untreated hosts ($P < 0.0005$). The maximal distance moved by the donor cells in antibody, antibody/thymus treated or untreated hosts was *2.2* mm, 2.3 mm or 1.0 mm respectively.

12 weeks

*12* weeks after grafting, in 6 TA muscles of antibody-treated hosts (mice #6, #7 and #8), 762 donor cells were

*Table 3. Distance moved by donor nuclei derived from sliced muscle grafts of male C57Bl/10Sn mice inserted into TA muscles of (A) antibody-treated, (B) antibody/thymus-treated or (C) untreated female mdx hosts: analysis with Y-1 probe* in situ.

| Mouse# | Sampling time | Longest distance moved by 10 nuclei (graticule units) | | Maximal Distance Moved | Average Distance Moved | Location of the Cells at the Maximal Distance |
|---|---|---|---|---|---|---|
| | | L TA | R TA | | | |
| | | A. Antibody-treated hosts | | | | |
| 1 | 1 week | 100, 97, 83. 80, 65. 64. 63. 60, 59, 50 | * | **0.8 mm** | 0.58 mm | between MF |
| 2 | 1 week | 137, 125. 101, 87, 84. 79. 77. 76. 76. 58 | ** | **1.1 mm** | 0.59 mm | within MF |
| 3 | 3 weeks | **100**. 99. 98. 98, 81, 76, 70, 60, 48. 43 | *** | **0.8 mm** | 0.61 mm | within MF |
| 4 | 3 weeks | 275. 205. 204. 203. 137. 137, 137, 137, 129, 129 | 275. 205. 204. 203. 137, 137. 137, 137, 129, 129 | **2.2 mm** | 1.24 mm | within MF |
| 5 | 3 weeks | * | 187, 111, 108. 105. 106. 107. 101, 100, 79, 81 | **1.5 mm** | 0.87 mm | within MF |
| 6 | 12 weeks | **252**. 250. 251. 251. 250. 251. 146. 121, 125, 127 | 221. 174. 166, 124. 121. 97. 101. 67. 67. 69 | **2.0 mm** | 1.29 mm | either |
| 7 | 12 weeks | 200. 200. 180, 110. 110, 105, 106. 91, 83, 79 | 235, 235, 179. 180. 179. 140. 131. 91. 92. 93 | **1.9 mm** | 1.06 mm | between MF |
| 8 | 12 weeks | 264. 263. 262. 204. 190, 173, 153, 110. 103, 89 | 202, 154. 122. 121. 120. 121. 112. 112. 101, 105 | **2.1 mm** | 1.2 mm | within MF |
| | | B. Antibody/thymus-treated hosts | | | | |
| 9 | 1 week | **137**. 137. 135. 135. 104. 105. 95, 94, 90, 75 | 120. 98. 98. 99. 78, 101, 102. 120. 119. 118 | 1.1 mm | 0.85 mm | within MF |
| 10 | 1 week | **40**. 35. 36. 36. 32. 33. 33. 37. 38. 39 | 39. 38. 39. 31. 31. 32. 20. 20, 26, 28 | 0.3 mm | 0.27 mm | within MF |
| 11 | 3 weeks | 112. 112. 109. 110. 110. 112. 112. 85. 84. 86 | * | **0.9 mm** | 1.04 mm | within MF |
| 12 | 3 weeks | **225**. 225. 225. 224, 202, 138, 98, 89, 78, 70 | 201. 170. 160. 158. 157. 157. 155. 141. 142. 120 | 1.8 mm | 1.25 mm | between MF |
| 13 | 3 weeks | 218. 193. 194. 193. 130. 130. 131. 132. 70, 75 | 287. 240. 240. 181, 168, 140, 100, 100, 101, 95 | 2.3 mm | 1.25 mm | within MF |
| 14 | 12 weeks | **256**, 214, 190, 161, 125, 126, 120, 112, 107, 102 | 195, 194, 194, 122, 123, 125. 95. 94. 95. 95 | 2.0 mm | 1.10 mm | either |
| 15 | 12 weeks | ** | **254**. 253. 251. 221. 160, 160, 140, 105, 104. 93 | **2.0 mm** | 1.39 mm | between MF |
| | | C. Untreated (control) hosts | | | | |
| 16 | 1 week | 75. 74. 74, 69, 68, 71. 72. 71. 70. 70 | 72. 73. 73. 69, 69, 67, 70, 65, 64, 64 | **0.6 mm** | 0.57 mm | within MF |
| 17 | 1 week | 132, 125, 110. 102. 102. 80. 80, 70, 50, 45 | 128. 114. 115. 115. 116. 91. 90. 89. 85, 80 | **1.1 mm** | 0.66 mm | between MF |
| 18 | 3 weeks | 100. 100. 98. 99. 90, 89, 77, 76, 70, 61 | 94, 90, 91, 88, 85, 83. 69, 62, 60, 60 | **0.8 mm** | 0.66 mm | within MF |
| 19 | 3 weeks | 125, 99. 98. 98. 81, 79, 74, 64, 59. 59 | 105, 100, 97. 93, 80, 80, 75, 63, 64, 55 | **1.0 mm** | 0.66 mm | within MF |
| 20 | 12 weeks | **132**, 111. 104. 105. 102, 100, 98, 69, 58, 55 | ** | **1.1 mm** | 0.75 mm | within MF |
| 21 | 12 weeks | 162, 153, 118, 116, 109. 74. 69. 67. 61. 60 | 178, 142, 131, 104, 92, 90, 89, 88, 71, 59 | **1.4 mm** | 0.82 mm | within MF |

NOTE: *Graft was not found; "Graft was found on surface of TA muscle; ***Y-1 probe hybridisation positive data, but not clear with respect to graft position; MF: myofibre(s); L TA: left TA muscle; R TA: right TA muscle; This represents the number of division on a graticule microscope eye piece, where division is 0.008mm. Where male nuclei were clustered together this is indicated by *underlining*. Bold type shows the maximum distance observed for both legs.

found, with 441 of them within and 238 between host muscle fibres (Fig. 4C). The maximal distance moved by these cells was 2.1 mm. In 3 TA muscles of hosts pre-treated with antibodies and thymus injection (mice #14 and #15), 449 donor cells were found, with 255 of them within and 106 between, host muscle fibres. The maximal distance moved by these cells was 2.0 mm. In 3 TA muscles of untreated hosts (mice #20 and #21), 79 donor cells were found, with 49 of them within and 14 between host muscle fibres (Fig. 4D). The maximal distance moved by these cells was 1.4 mm.

In antibody and antibody/thymus treated hosts, there was no difference in the survival of donor myoblasts at any time during the experiments. The average numbers of migrated donor cells for the antibody treated hosts at 1 week, 3 weeks and 12 weeks were 157, 113 and 127 ($P > 0.05$). The average numbers of migrated donor cells for the antibody/thymus treated hosts at 1 week, 3 weeks and 12 weeks were 103, 107 and 149 ($P > 0.05$). In untreated hosts, the numbers of surviving donor cells were significantly reduced at 12 weeks compared with 1 week after grafting (average of 56 and 23 cells at 1 and 12 weeks respectively, $P < 0.05$). This difference was not found between 3 and 12 weeks within all three groups (average of 22 and 23 cells at 3 and 12 weeks respectively, $P > 0.05$).

12 weeks after grafting the distance moved by the cells was different between antibody treated and untreated hosts (average distance moved was 1.18 mm and 0.79 mm, $P < 0.0005$), and antibody/thymus treated and untreated hosts (average distance moved was 1.25 mm and 0.79 mm, $P <$

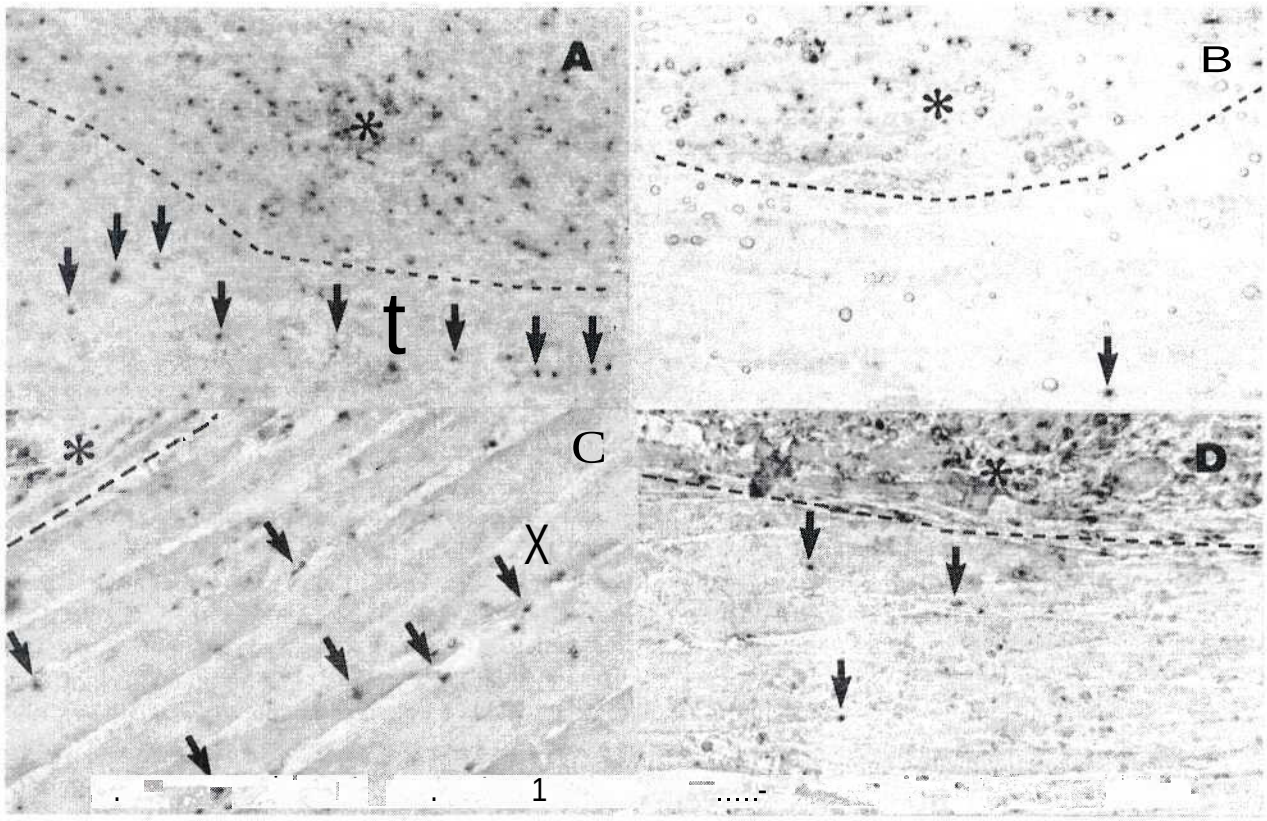


*Figure 4. Y-1 probe* in situ *analysis of grafted muscle tissues. Sliced male host C57Bl/10Sn muscles (*, above broken line) were grafted into the TA muscles of the (A, C) antibody-treated (Table 2. Group A) or (B, D) untreated female mdx mice (Table 2, Group C). The muscles were sampled at three (A, B) or twelve (C, D) weeks after grafting and hybridised with the Y-l probe. The nuclei of the female host muscle do not hybridise with the Y-l probe which only detects male nuclei (clearly demonstrated in B). Male donor nuclei (some examples shown by arrows) had moved out from the grafts into the female host muscles. Magnification of all panels is x400.*

0.0005), but not between antibody and antibody/thymus treated hosts.

It was found that most of these male donor nuclei that had moved into the host muscles were located within myofibres and there were relatively few lying outside myofibres in all 3 groups ($P < 0.001$).

**Discussion**

The survival and movement of donor myoblasts out from sliced muscle grafts and their fusion with host myofibres was significantly enhanced in T-cell depleted hosts, and T-cell depleted and tolerised hosts, compared with untreated hosts. A decrease of over 50% in the number of surviving, migrating donor myoblasts was seen in the untreated hosts over the 3 week period and continued to 12 weeks after grafting. In contrast, this decrease was not seen in T-cell depleted or T-cell depleted/tolerised hosts: (the average numbers of migrated cells at 1:3:12 weeks for the antibody treated only, antibody/thymus treated and untreated hosts, were 157:113:121, and 103:107:149 compared with 56:23:26 respectively). Furthermore, the maximal distance moved by the donor cells of T-cell depleted and T-cell depleted/tolerised hosts was 2.2 mm and 2.3 mm at 3 weeks, 2.1 mm and 2.0 mm at 12 weeks, compared with a distance of only 1.0 mm and 1.4 mm for untreated hosts at 3 and 12 weeks in the present study and 1.6 mm at 3 weeks in an earlier study [12]. These results indicate that T-cell depletion either alone or combined with the induction of antigen tolerance in the thymus, significantly increased the survival of normal donor myoblasts in the dystrophic host muscle. The host treatments appeared to have the maximal effect on cell survival between 1 and 3 weeks because the numbers of surviving donor cells were only significantly reduced between 1 and 3 weeks after grafting in the untreated hosts.

The treatment of host mice also enhanced the emigration of donor myoblasts from sliced muscle grafts into host muscle in comparison with untreated hosts. This might be due to a direct affect on cell movement and/or reflect the increased myoblast survival. The fact that more donor nuclei were located within muscle fibers, rather than between muscle fibres, suggests that the fusion of donor myoblasts with host myofibres protects the donor cells in the host environment.

From the data presented here, and that presented by others [51], it seems clear that T-cells, $CD4^+$, $CD8^+$ or both, play a role in determining the survival of syngeneic myoblasts after transplantation. The initiating events are

unclear and the degree of antigen specificity is also not known. It is possible that the rejection of the C57Bl/10Sn myoblasts reflects recognition of dystrophin as a foreign antigen by the host *mdx* mice which are dystrophin deficient. Although, the presence of revertant fibres in these mice [26] indicates that they would have been exposed previously to the vast majority of dystrophin epitopes, the low level of peripheral expression of this antigen may prevent the development of tolerance.

Alternatively, the interaction between T-cells and the incoming syngeneic myoblasts may reflect a non-antigen specific interaction. T-cells have been reported to infiltrate degenerating/regenerating skeletal muscle in Duchenne muscular dystrophy [1] and after exercise-induced muscle damage [40]. Certain molecules expressed by myoblasts may be chemotactic or mitogenic for T-cells and may promote adherence of T-cells to myoblasts. Myoblasts can secrete IL-6 [3], and perhaps other cytokines, which may contribute to the inflammatory process in implanted muscle. Adherence between T-cells and autologous myoblasts has been observed under *in vitro* culture conditions [5, 16] and this interaction was increased when myoblasts were exposed to cytokines which upregulate the expression of adhesion molecules such as ICAM-1 and when T-cells were activated [5]. Intimate membrane-membrane interactions are inferred by the transfer of lysosomal enzymes between T-cells and myoblasts in culture [4]. The environment of the damaged muscle may be sufficient to non-specifically activate T-cells which may directly damage the myoblasts or may recruit and activate macrophages, with resultant myoblast destruction. The important role for $CD8^+$ T- cells in the inflammatory responses seen in 4 week old *mdx* mice would again support a role for non-antigen specific interaction between T-cells and muscle cells during inflammatory responses [43].

The observation that induction of T-cell tolerance by thymic injection did not appear to give any advantage over antibody depletion alone in transplanted muscles studied for up to 12 weeks is consistent with, although does not prove, a non-antigen specific mechanism. From a practical viewpoint it suggests that this additional manipulation may not be necessary although it is possible that a difference between the two groups might become apparent in long-term studies.

The marked improvement in MTT by immunomanipulation of the adult host animals provides a powerful new approach for optimising the conditions for successful MTT. In theory, such T-cell depletion and induction ofhost tolerance could be readily applied to the clinical situation. This represents a highly attractive alternative to long-term drug immunosuppression of hosts with the undesirable side-effects.

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