Transient Immunosuppressive Treatment Leads to Long-term Retention of Allogeneic Myoblasts in Hybrid Myofibers

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Abstract. Normal and genetically engineered skeletal muscle cells (myoblasts) show promise as drug delivery vehicles and as therapeutic agents for treating muscle degeneration in muscular dystrophies. A limitation to the widespread use of myoblast transplantation is the immune response of the host to the transplanted cells. Allogeneic myoblasts are rapidly rejected unless immunosuppressants are administered. However, continuous immunosuppression is associated with significant toxic side effects. Here we test whether immunosuppressive treatment, administered only transiently after allogeneic myoblast transplantation, allows the long-term survival of the transplanted cells in mice. Two immunosuppressive treatments with different modes of action were used: (a) cyclosporine A (CSA); and (b) monoclonal antibodies to intracellular adhesion molecule-1 and leukocyte function-associated molecule-1. The use of myoblasts genetically engineered to express β-galactosidase allowed quantification of the survival of allogeneic myoblasts at different times after cessation of the immunosuppressive treatments. Without host immunosuppression, allogeneic myoblasts were rejected from all host strains tested, although the relative time course differed as expected for low and high responder strains. The allogeneic myoblasts initially fused with host myofibers, but these hybrid cells were later destroyed by the massive immunological response of the host. However, transient immunosuppressive treatment prevented the hybrid myofiber destruction and led to their long-term retention. Even four months after the cessation of treatment, the hybrid myofibers persisted and no inflammatory infiltrate was present in the tissue. Such long-term survival indicates that transient immunosuppression may greatly increase the utility of myoblast transplantation as a therapeutic approach to the treatment of muscle and nonmuscle disease.

Myoblasts are among the leading candidates for cell-mediated gene therapy (Blau et al., 1993; Partridge, 1994). Myoblasts are proliferating precursor cells found in skeletal muscle tissue which upon differentiation fuse with one another to form multinucleated muscle fibers. Transplanted myoblasts fuse with endogenous muscle fibers and are maintained in a stable physiological environment for extended periods in vivo (Hughes and Blau, 1992; Rando and Blau, 1994). Myoblasts have been tested as drug delivery vehicles in animals (Barr and Leiden, 1991; Dhaawan et al., 1991; Dai et al., 1992; Jiao et al., 1993) and as therapeutic agents in the treatment of muscle degeneration in human muscular dystrophies (Gussoni et al., 1992; Law et al., 1992; Karpati et al., 1993; Tremblay et al., 1993b).

A limitation to the widespread use of myoblast transplantation is the immune response of the host. Myoblasts transplanted across histocompatibility barriers are rapidly rejected in immunocompetent hosts (Watt, 1990; Labrecque et al., 1992; Huard et al., 1994; Rando and Blau, 1994). Current drugs used clinically to prevent rejection of foreign tissue transplants must be taken lifelong and have toxic side effects. The discovery of alternate strategies of host immunosuppression which are transient but lead to the long-term maintenance of allogeneic myoblasts would facilitate the use of myoblasts for gene therapy purposes.

Transient immune suppression around the time of transplantation has led to long-term survival of allografts such as organs, skin, and neural cells. Transient treatments have included short term administration of immunosuppressive drugs such as cyclosporine A (Green and Allison, 1978; Calne et al., 1979; Green et al., 1979; Nagao et al., 1982; White and Lim, 1988; Auchenluss and Winn, 1989; Ortega et al., 1992), which block the production of cytokines necessary for T cell activation (Borel, 1990) or monoclonal antibodies against cell surface molecules such as CD2, CD3, CD4, CD8, intracellular adhesion molecule-1 (ICAM-1), 1

Abbreviations used in this paper: β-gal, β-galactosidase; CSA, cyclosporine A; H&E, hematoxylin and eosin; ICAM-1, intracellular adhesion molecule; LFA-1, leukocyte function-associated molecule-1; MHC, major histocompatibility complex; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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The Journal of Cell Biology, Volume 127, Number 6, Part 2, December 1994 1923-1932 1923
leukocyte function-associated molecule-1 (LFA-1), and LFA-3 (Martz, 1987; Waldmann, 1989; Waldmann and Cobbold, 1993), which are involved in immune recognition and function (Roitt, 1991). Treatment with monoclonal antibodies can induce long-term unresponsiveness or tolerance in specific immune cells which interact with the target tissue early after transplantation. This is termed antigen-specific tolerance.

Transient immunosuppression may be efficacious for long-term survival of transplanted myoblasts. The expression of class I and II major histocompatibility complex (MHC) molecules, which are critically involved in immune rejection, is regulated in muscle differentiation. MHC molecules are normally expressed by myoblasts (Honda and Rostami, 1989; Cifuentes-Diaz et al., 1992; Hardiman et al., 1993a), but upon fusion and differentiation, muscle cells generally do not express MHC molecules (Ponder et al., 1983; Karpati et al., 1988b; Honda and Rostami, 1989; Schubert, 1991). Only in diseased and regenerating muscle biopsies are MHC molecules expressed by myofibers (Rowe et al., 1983; Appleyard et al., 1985; Karpati et al., 1988b; Emslie-Smith et al., 1989; McDouall et al., 1989). Within five days of transplantation, syngeneic and allogeneic myoblasts fuse with endogenous fibers of the host (Rando and Blau, 1994). Upon incorporation into endogenous myofibers, myoblast nuclei are subject to trans-activating muscle regulatory factors within the differentiated muscle cell (Blau et al., 1983, 1985) and may cease expression of MHC molecules. Thus, we postulated that if the immune system could be transiently suppressed during the period of cell fusion, the foreign myoblasts that fused might be hidden from the immune system in the "immunologically silent" myoblast.

In this study we test the hypothesis that transient immune suppression is efficacious for the long-term retention of allogeneic myoblasts in hybrid myoblasts. The use of myoblasts genetically engineered to express β-galactosidase (β-gal) allowed histological assessment of the fate of the transplanted myoblasts after transplantation. Without immunosuppression, allogeneic myoblasts were rejected from all host strains tested, although the relative time course differed indicating the existence of low and high responder strains. Rejection of allogeneic myoblasts was successfully overcome with transient administration of two immunosuppressive treatments with different modes of action: (a) cyclosporine A and (b) monoclonal antibodies to ICAM-1 and LFA-1. Hybrid myoblasts were retained for more than four months after the cessation of immune suppressive treatment. These findings indicate that transient immunosuppressive treatment is effective for long-term retention of allogeneic myoblasts and should have practical applications to myoblast-mediated gene therapy.

Materials and Methods

Animals

Adult male mice (2.5-5 wk old) of the BALB/c, C57BL/6, and C3H/Km strains were purchased either from the Department of Radiology at Stanford University or from Simonsen Laboratories, Inc. (Gilroy, CA) and housed in viral- and pathogen-free conditions. The haplotype of the MHC genes in the H-2 complex of these strains is H-2d (BALB/c), H-2b (C57BL/6), and H-2k (C3H/Km). Donor and recipient pairs were fully mismatched at both major and minor histocompatibility loci. All animals were handled in accordance with guidelines of the Administrative Panel on Laboratory Animal Care of Stanford University.

Cell Culture

Primary muscle cultures were derived from neonatal mice (2-5-d-old) of all three strains indicated above and myoblasts were purified to >99%, as previously described (Rando and Blau, 1994). Cells were grown in Ham's F-10 media (GIBCO BRL, Gaithersburg, MD) supplemented with 20% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 2.5 mg/ml basic fibroblast growth factor (Promega Corp., Madison, WI), penicillin G (200 units/ml), and streptomycin (200 μg/ml) on collagen-coated dishes in a humidified 5% CO2 incubator at 37°C. Cultures were infected with the replication defective retroviruses BAG (Price and Thurlow, 1988) or c-SCG (Dawani et al., 1991) which express β-gal and enriched to >95% β-gal positive either by selection with media containing G418 (for BAG-infected cells) or by flow cytometry as previously described (Rando and Blau, 1994). Donor myoblasts were 95-99% positive for β-gal expression at the time of transplantation as measured by in vitro staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sanes et al., 1986).

Immunosuppression

Cyclosporine A (CSA). CSA (Sandimmune IV; Sandoz, East Hanover, NJ) was administered by daily i.p. injections: 70 mg/kg BALB/c, 75 mg/kg C57BL/6, 55 mg/kg C3H/Km. The dose of CSA was empirically determined as the highest concentration which resulted in no animal mortality in the first week when administered daily. A stock solution of 10 mg/ml in sterile PBS was prepared fresh weekly and stored at 4°C. Animals were treated with CSA for 2 wk, 6 wk, or continuously as indicated in particular experiments. CSA-treated animals were maintained on antibiotic water containing 1.1 mg/ml neomycin (Sigma Chem. Co., St. Louis, MO) and 850 units/ml polymixin B sulfate (Pharm-Tek, Huntington, NY).

Monoclonal Antibodies. Monoclonal antibodies against ICAM-1 (YIN/1.74, rat IgG2a; Prieto et al., 1989) and LFA-1 (PD441.8, rat IgG2a; Sarmiento et al., 1982) were purified from hybridoma supernatants. The hybridomas were obtained from the American Type Culture Collection (Rockville, MD) and grown in serum-free HB101 (Irvine Scientific, Irvine, CA) for 6-9 d. The media was concentrated by ultrafiltration using a YM100 membrane (Amicon, Beverly, MA) and the antibodies were purified using a protein G column (Mab Trap G; Pharmacia, Piscataway, NJ). The total protein concentration was determined (Bradford, 1976) and the activity of different preparations was standardized using staining of single cell suspensions of the spleen from mice and analysis by flow cytometry. Animals were injected daily i.p. with a 0.2 ml mixture of the purified antibodies (140 μg each) in PBS for a total of 6 d starting on the date of myoblast transplantation. Peripheral blood (~0.15 ml) obtained from the tail vein at 10 and 28 d after transplantation was diluted 1:6 in PBS containing heparin and the lymphocytes recovered using Lympholyte M (Accurate Chemicals, Westbury, NY). In order to stain and quantitate the cells bearing the αICAM-1 and αLFA-1 rat antibodies used for treatment, peripheral blood lymphocytes were incubated with fluorescein-conjugated goat antibody to rat immunoglobulin (adsorbed with mouse immunoglobulin; Caltag, San Francisco, CA) for 30 min at 4°C in staining buffer consisting of 2% heat-inactivated calf serum and 0.1% sodium azide in PBS. The cells were then washed two times and resuspended in staining buffer with 1 μg/ml propidium iodide. Samples were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer equipped with logarithmic amplifiers. Dead cells were identified by their staining with propidium iodide and eliminated from the analysis.

Cell Transplantation

Cultured cells were trypsinized and washed several times in transplantation buffer which consisted of 0.5% bovine serum albumin in F10. The cells were resuspended in transplantation buffer at a density of 2 × 10^7 cells/ml and kept on ice. Animals were anesthetized with an i.p. injection of sodium pentobarbitol (~60-80 mg/kg depeding on the age and strain of the mice). Two inguinal cell suspension of 5 μl each were made into each tibialis anterior muscle as previously described (Rando and Blau, 1994). In the experiments testing transient immunosuppressive treatment with monoclonal antibodies, one 5-μl injection of the cell suspension was made into the lateral muscles of the hind limb through a shaved portion of the intact skin without microscopic visualization.
Fate of Implanted Myoblasts

Mice were sacrificed at different times after transplantation. The injected muscles were removed and prepared for histological analyses as previously described (Rando and Blau, 1994). Serial cross sections collected at 400-500-μm intervals along the entire length of the muscle were analyzed for β-gal expression by staining with X-gal or for histology by hematoxylin and eosin (H&E) staining. The number of β-gal-positive muscle fibers in an individual leg was scored as the value obtained in the cross section with the greatest number of labeled fibers. For all groups (3-10 samples), the mean numbers of β-gal-labeled fibers ± SEM were calculated. All analyses and photography was performed on a Zeiss Axiophot microscope.

Results

Fate of Allogeneic Myoblast Transplants without Immunosuppression in Different Host Strains

The immune response to purified antigens and organ transplants as well as the efficacy of immunosuppressive treatments can vary significantly among rodent strains (Butcher and Howard, 1982; Stewart et al., 1985; Sprent et al., 1986; Rosenberg et al., 1987; Ilano et al., 1989). As a control for subsequent studies of transient immunosuppression, the time course of allogeneic myoblast rejection in different strains of mice was first established. We have previously shown that β-gal–labeled primary myoblasts are retained in hybrid myofibers for many months in syngeneic or immunodeficient hosts (Rando and Blau, 1994). In contrast, allogeneic myoblasts are rejected by 2–3 wk in the C3H host. To extend these observations to other mouse strains, pure cultures of myoblasts from different haplotype donors were labeled with replication-defective retroviruses expressing β-gal and transplanted into three different host strains. The retention or rejection of the transplanted cells was determined by counting the number of β-gal–labeled fibers in the hosts at different times after transplantation. As controls, the cells were also transplanted into the muscles of immunodeficient mice.

At two and four weeks after transplantation, the number of β-gal–labeled fibers in cryostat sections from the tibialis anterior was determined for each donor–host combination. Differences were observed in the kinetics of rejection of allogeneic myoblasts in different donor-host combinations without any immunosuppressive treatment (Table I). Two weeks after transplantation in C57 and C3H hosts, only 2% of the number of labeled fibers remained as compared to that obtained in immunodeficient hosts. In contrast, in BALB/c hosts transplanted with C3H or C57 myoblasts, 5 and 23% of the control number of β-gal–labeled fibers, respectively, were obtained at this time. However, analyses of BALB/c hosts 4 wk after transplantation revealed no β-gal–labeled fibers. Histologically, the rejection process of all allogeneic myoblasts was similar. First, allogeneic myoblasts fused normally with host myofibers by day 5 (Rando and Blau, 1994; Fig. 1, top). However, by 10 d, a prominent host mononuclear cell infiltrate was present (Fig. 1, middle left) which destroyed the hybrid muscle fibers leading to disruption of the normal architecture of the muscle and a decrease in the number of β-gal–labeled fibers (Fig. 1, middle right). As in other situations in which muscle injury occurs, this region of muscle fully regenerated (Fig. 1, bottom left) but no β-gal–labeled fibers were present (Fig. 1, bottom right). Thus, allogeneic myoblasts were rejected in all donor–host combinations, although the relative time course differed somewhat. In all strains, the ensuing local destruction of host myofibers was followed by normal regeneration. These studies indicate that drugs used for immunosuppression in other situations, that “low” and “high” responder rodent strains exist (Butcher and Howard, 1982; Stewart et al., 1985; Sprent et al., 1986; Rosenberg et al., 1987; Ilano et al., 1989) that mount an immune response with different time courses.

The Effect of Continuous CSA on Maintenance of Allogeneic Myoblasts

CSA has been used to block organ allograft rejection in numerous experimental and clinical protocols. Similarly, in the current experiments daily treatment with CSA prevented the rejection of allogeneic myoblasts in all donor–host combinations tested. Hybrid myofibers composed of allogeneic myoblasts fused with host myofibers, as evidenced by large β-gal–labeled fibers (Fig. 2, top) with normal histology (Fig. 2, bottom), were maintained in CSA–treated animals for more than two months with no evidence of immune rejection. To test the efficacy of CSA treatment, we compared the number of hybrid fibers formed from the transplantation of myoblasts into CSA-treated allogeneic hosts to the number formed from the transplantation of the same cells into immunodeficient hosts. There was no significant difference (Fig. 3). Thus, CSA efficiently suppressed the immune response to allogeneic myoblasts.

Others have reported that drugs used for immunosuppressive treatment may directly effect myoblast fusion in vitro (Hardiman et al., 1993b). To study the effect of CSA on myoblasts in vivo independent of its immunosuppressive action, β-gal–labeled myoblasts were transplanted into CSA treated and untreated syngeneic hosts. The number of β-gal–labeled fibers observed 2 wk after transplantation did not differ between control and CSA treated animals (Fig. 4). Therefore, in contrast to in vitro results (Hardiman et al., 1993b) CSA even at maximally tolerated doses did not appear to inhibit myoblast fusion in vivo.
Allogeneic myoblasts are rejected without immunosuppressive treatment. β-gal–labeled myoblasts were transplanted into allogeneic mice and examined at different timepoints thereafter. Muscles were frozen and cryostat sections were prepared for analysis. Adjacent cross sections were stained with H&E to demonstrate histology (left) and with X-gal to identify the location of the hybrid muscle.
CSA-treated animals exhibit similar retention of hybrid myofibers as do immunodeficient animals. β-gal-labeled C57 myoblasts were transplanted into either immunodeficient scid (●) or C3H hosts. C3H hosts transplanted with these allogeneic myoblasts received either daily injections of CSA (▼) or were untreated (▲). Three and a half to four weeks after transplantation, the number of β-gal-labeled fibers in each injected muscle was determined. The mean number of labeled fibers in the scid hosts was defined as 100% (79 ± 8) and the mean numbers of fibers in CSA-treated and untreated C3H hosts were compared to this value. (error bars = ±SEM; n = 7).

Figure 2. Continuous immunosuppressive treatment with CSA allows long-term retention of allogeneic myoblasts in hybrid myofibers. β-gal–labeled myoblasts were transplanted into allogeneic mice and the hosts treated with CSA daily. The hybrid myofibers formed (large diameter β-gal–labeled fibers) were maintained for more than two months (top). Complete suppression of the host immune response observed in untreated controls (Fig. 1) was revealed by standard H&E staining of the adjacent cross section (bottom). Bar, 60 μm.

Transient CSA Treatment: Prevention of Allogeneic Myoblast Rejection

To test the hypothesis that transient immunosuppression during the initial period of myoblast transplantation, fusion, and maturation prevents the rejection of allogeneic myoblasts, β-gal–labeled C57 myoblasts were transplanted into BALB/c or C3H hosts and the animals received daily injections of CSA starting on the day of transplantation. Two weeks of fibers by β-gal activity (right). 5 d after transplantation, no inflammatory cell infiltrate was observed (top, left) and many allogeneic myoblasts had fused into endogenous host myofibers (top, right). 10 d after transplantation, the rejection process was characterized by a host mononuclear cellular infiltrate and local tissue destruction (middle, left) in the region of β-gal–labeled fibers (middle, right). By 4 wk after transplantation, the host cellular immune response had abated and the muscle fibers had regenerated (bottom, left). Centrally nucleated muscle fibers, a permanent marker of muscle fiber regeneration in rodents, were present. No β-gal–labeled fibers remained in animals without immunosuppression (bottom, right). Bar, 60 μm.

Figure 4. CSA does not affect the number of β-gal–labeled fibers formed from transplanted myoblasts in syngeneic hosts. β-gal–labeled myoblasts were transplanted into syngeneic hosts. Both BALB/c and C57 hosts were used. The animals were either untreated (●) or treated (▼) with CSA. 2 wk after transplantation the number of β-gal–labeled fibers in each injected muscle was determined. The mean number of labeled fibers in untreated hosts was defined as 100% (BALB/c: 141 ± 19; C57: 200 ± 14) and the mean number of fibers in CSA-treated and untreated C57 hosts was compared to this value. (error bars = ±SEM; n = 4).
**Figure 5.** Transient immunosuppressive treatment with CSA leads to long-term retention of allogeneic myoblasts in hybrid myofibers in BALB/c or C3H hosts. Long-term survival of allogeneic myoblasts is obtained with a short course of CSA treatment during the early times after myoblast transplantation and fusion into endogenous fibers. The persistence of β-gal-labeled fibers with time indicates the lack of a functional host immune response to the hybrid myofibers. β-gal-labeled C57 myoblasts were transplanted into either BALB/c (●) or C3H (■) hosts. Daily injections of CSA were administered starting on the day of transplant, continued for 2 wk and then stopped. At different times after stopping CSA treatment, the animals were sacrificed and the number of β-gal-labeled fibers was determined for each injected muscle. For each host strain, the mean number of fibers in CSA treated animals at the time of CSA removal was defined as 100% (BALB/c: 186 ± 33; C3H: 80 ± 19). The mean number of fibers at different times after CSA removal were compared to this value. (error bars = ±SEM; n = 4–8).

CSA administration was chosen as the length of immunosuppressive treatment for two reasons. First, the number of hybrid myofibers formed does not increase significantly beyond 5 d after transplantation (Rando and Blau, 1994). Second, the local muscle regeneration that is a consequence of myoblast transplantation is nearly complete within 2 wk and this is accompanied by a corresponding decrease in the expression of fetal myosin heavy chain, a marker of regenerating muscle fibers (Pavlath and Rando, unpublished observations). Treatment with the immunosuppressive drug was stopped after 2 wk and the stability of the hybrid β-gal-labeled fibers was studied for an additional 14–16 wk. Transient treatment with CSA was efficacious for the long-term retention of allogeneic myoblasts in both strains (Fig. 5). 14–16 wk after the cessation of CSA, or 4–4.5 mo after the initial transplant, the numbers of β-gal-labeled fibers in BALB/c and C3H hosts were 95 and 80%, respectively, of control values (Fig. 5). The histology of the muscle tissue at these late time points was indistinguishable from that seen with continuous CSA. β-gal expression was found in large diameter fibers similar in size to the surrounding muscle tissue (Fig. 6, top) with no mononuclear infiltration present in the muscle (Fig. 6, bottom). Thus, transient immunosuppressive treatment with CSA resulted in long-term retention of fully MHC mismatched myoblasts in BALB/c and C3H hosts with no evidence of a host cellular immune response.

**Figure 6.** Long-term retention of hybrid myofibers with transient CSA treatment is accompanied by normal muscle histology. Serial cross sections analyzed for β-gal expression (top) and histology (bottom) are shown from animals transplanted with allogeneic β-gal-labeled myoblasts, treated with CSA for 2 wk and sacrificed 16 wk after cessation of CSA treatment. β-gal expression is found in large diameter, mature muscle fibers. No host cellular immune response is present. Bar, 60 μm.

**Transient CSA Treatment: Delay of Rejection vs. Long-term Maintenance**

Transient CSA treatment was also tested in the C57 strain. The efficacy of transient CSA treatment, however, differed in this host. β-gal-labeled C3H myoblasts were transplanted into C57 hosts and the animals treated with daily injections of CSA for 2 wk. Unlike the success of transient CSA immunosuppressive treatment for the long-term retention of allogeneic myoblasts in the BALB/c and C3H hosts, 2 wk was not sufficient in the C57 strain (Fig. 7, black bars). Only 8% of β-gal-labeled fibers remained 2–5 wk after the cessation of CSA treatment. We hypothesized that a longer period of CSA treatment might be necessary in this host. However, 6 wk of CSA treatment (Fig. 7, hatched bars) delayed, but did not eliminate the rejection of allogeneic myoblasts. Following 6 wk of CSA treatment, the number of β-gal-labeled fibers declined to 38% 2–5 wk later and to 15% 8–12 wk later. Thus, transient immunosuppression with CSA did not lead...
to long-term retention of fully MHC mismatched myoblasts in the C57 strain, but delayed the usual rejection.

**Immunosuppression with Monoclonal Antibodies to T Cell Molecules**

Accessory molecules, such as ICAM-1 and LFA-1, increase the adhesion between T cells and their target cells and provide necessary co-stimulatory signals for T cell activation (Springer et al., 1987; Springer, 1990). Limited treatment of the host (6-12 d) with monoclonal antibodies against ICAM-1 and/or LFA-1 leads to prolonged survival of organ and bone marrow allografts in animals (Heagy et al., 1984; Benjamin et al., 1988; Cosimi et al., 1990; Charlton et al., 1991; Isobe et al., 1992) and humans (Fischer et al., 1986; Haug et al., 1993). We tested whether transient treatment with antibodies against ICAM-1 and LFA-1 was more effective than transient CSA treatment in preventing rejection of allogeneic myoblasts in C57 hosts. For comparison, we tested transient antibody treatment in a strain (C3H) in which transient CSA treatment had led to long-term retention of allogeneic myoblasts. For both groups we used continuous CSA treatment as a control. For the antibody treatment groups, the animals were injected daily with 140 μg each of antibodies to ICAM-1 and LFA-1 for 6 d beginning on the day of transplantation. The levels of the injected rat antibodies on the peripheral blood lymphocytes were monitored by flow cytometry. Saturating levels of antibodies were observed up to 10 d, with intermediate levels ~16 d, but were undetectable by ~3 wk after transplantation.

In C3H hosts, transient treatment with the antibodies, like transient CSA treatment, was as effective as continuous CSA treatment in preventing the rejection of allogeneic myoblasts (Fig. 8 A). In C57 hosts, in which transient CSA treatment was ineffective, transient treatment with antibodies against ICAM-1 and LFA-1 was as effective as continuous CSA allowing long-term retention of allogeneic myoblasts (Fig. 8 B). These studies indicate that maintenance of allogeneic myoblasts in hybrid myofibers using transient immunosuppressive treatments is a generalized phenomenon. The efficiency in different genetic backgrounds may depend on the specific mode of treatment.

**Discussion**

Gene delivery by the transplantation of normal and genetically engineered myoblasts is the basis of a new experimental approach to the therapy of muscle and nonmuscle diseases. Ideally, the myoblasts used for gene delivery should have extensive proliferative capacity in culture, be readily available in large quantities and be nontumorigenic. As we have shown previously, these requirements can be met (Webster et al., 1988; Rando and Blau, 1994). Two possibilities exist as sources of myoblasts for transplantation: autologous or heterologous donors. Generating autologous myoblasts for each patient severely limits this therapeutic approach because it is slow and labor intensive. Furthermore, myoblasts isolated from patients affected with Duchenne muscular dystrophy have a limited proliferative capacity in culture (Blau et al., 1983; Webster and Blau, 1990). Alternatively, the use of a normal identical twin as a donor for a sibling with muscle disease is limited to only a few special circumstances (Trem...
blay et al., 1993a). Thus, the ability to use myoblasts from a "universal donor" would increase the general utility of myoblast transplantation.

Heterologous myoblasts, like organ transplants, are rapidly rejected unless immunosuppressants are administered (Rando and Blau, 1994). CSA is the most prevalent immunosuppressive drug used in tests of allogeneic myoblast transplants in animals (Watt et al., 1981, 1984; Salminen et al., 1991; Labrecque et al., 1992; Huard et al., 1994) and humans (Gussoni et al., 1992; Law et al., 1992). However, continuous administration of the drug results in significant animal mortality (Watt et al., 1981, 1984; Huard et al., 1994) which is host strain and age specific (Pavlath and Rando, unpublished observations). Furthermore, chronic administration of immunosuppressive drugs may affect the clinical course of the muscle disease for which myoblast-mediated gene therapy is intended (Brooke et al., 1987; DeSilva et al., 1987; Mendell et al., 1989; Fenichel et al., 1991; Griggs et al., 1993; Sharma et al., 1993) or affect the properties of myoblasts such as their ability to fuse (Hardiman et al., 1995b). In addition, the lifelong administration of CSA and other immunosuppressive drugs in humans to prevent rejection of foreign organ transplants is associated with toxic side effects including depression of the immune system thereby increasing the risk of life threatening infection or cancer. Alternate strategies of immunosuppression which are transient but lead to the long-term maintenance of heterologous myoblasts are needed.

Myoblast transplantation poses unique challenges in comparison to organ transplants. In contrast to organs, transplanted myoblasts rapidly fuse into endogenous muscle fibers of the host by day 5 after transplantation. Interestingly, allogeneic myoblasts, like syngeneic myoblasts are also normally incorporated into the fibers of the host (Rando and Blau, 1994). Around day 7, the two types of cell transplants can be distinguished histologically. With allogeneic transplants, a massive mononuclear cellular infiltrate appears which attacks the hybrid myofibers leading to segmental muscle degeneration. In the studies presented here, the majority of the hybrid myofibers identified by histological staining for β-gal activity were destroyed by 2 wk after transplantation, although rejection was somewhat slower in one donor-host combination. However, in all host strains, the destroyed region of the myofiber segment regenerated resulting in normally sized, centrally nucleated myofibers, a hallmark of muscle regeneration (Karpati et al., 1988a). Thus, in contrast to allograft rejection of organs, the rejection of allogeneic myoblasts not only results in loss of the transplanted cells but also induces tissue damage and degeneration of previously healthy muscle fibers in the host.

The rapid rejection of allogeneic myoblasts was overcome using transient immunosuppressive treatments in mice. Two different transient treatments, CSA and monoclonal antibodies to ICAM-1 and LFA-1, led to persistent survival of allogeneic myoblasts in hybrid myofibers. However, strain differences among hosts were observed in the efficacy of these two treatments. The robust cell survival in BALB/c and C3H hosts treated with CSA for only 2 wk after transplantation contrasted with the limited survival of allogeneic myoblasts in C57 mice even with 6 wk of CSA treatment. However, administration of monoclonal antibodies starting on the day of transplantation and continuing for 5 d afterwards in C57 mice resulted in the same level of retention of allogeneic myoblasts as with continuous daily CSA administration. Therefore, the ability of transient immunosuppression to induce prolonged survival of allogeneic myoblasts in hybrid myofibers occurred in all strains tested. Although the method of transient immunosuppressive treatment differed, the outcome was the same. Other studies have noted strain differences in the efficiency of immunosuppressive treatments in rodents (Ilano et al., 1989) leading to the concept of low and high responder strains in different experimental paradigms of transplant immunology. Elucidation of the cellular and molecular bases for such genetic differences (Butcher and Howard, 1982; Stewart et al., 1985; Sprent et al., 1986; Rosenberg et al., 1987; Ilano et al., 1989) may contribute to an understanding of human variabilities in response to immunosuppressive drugs.

The mechanisms by which transient treatment with CSA or monoclonal antibodies to ICAM-1 and LFA-1 lead to persistent survival of allogeneic myoblasts in hybrid myofibers is unknown. Two mechanisms have been proposed for the ability of transient immunosuppressive treatment to prolong allograft survival: (a) changes in the immunogenicity of the graft (Auchincloss and Winn, 1989; Ortega et al., 1992) and (b) changes in the immune system leading to the development of peripheral tolerance (Hall, 1989; Waldmann, 1989; Waldmann and Cobbold, 1993). These two mechanisms have different implications for the fate of the original transplanted myoblasts after muscle injury as well as for the survival of subsequent myoblast transplants without further immunosuppressive treatment. These different mechanisms are directly testable in our system.

According to a mechanism based on altered immunogenicity of the transplant, the role of transient immunosuppression during early times after transplantation is to protect the myoblasts until they fuse into host myofibers and downregulate the expression of molecules involved in graft rejection. The immunogenicity of transplanted myoblasts is likely to change because the expression of molecules with critical roles in immune recognition of allografts declines during muscle differentiation (Ponder et al., 1983; Karpati et al., 1988b; Honda and Rostami, 1989; Schubert, 1991). Generally, the undifferentiated myoblast expresses molecules important in immune recognition but the differentiated myofiber of the host does not. After transplantation and subsequent fusion into endogenous myofibers, allogeneic myoblast nuclei may differentiate in response to regulatory factors provided by the mature myofiber (Blau et al., 1983, 1985) and may cease expression of foreign MHC molecules. Changes in the expression of these molecules would affect the cellular interactions between transplanted myoblasts and immune cells. An altered immunogenicity mechanism predicts any condition which induces MHC expression such as muscle damage (Rowe et al., 1983; Appleyard et al., 1985; Karpati et al., 1988b; Emslie-Smith et al., 1989; McDouall et al., 1989) would lead to rejection of the foreign cells. Furthermore, subsequent myoblast transplants would still require immunosuppression for long-term cell survival.

Alternatively, changes in the immune system may have also played a role in the success of transient immunosuppression in these studies. CSA is not ordinarily associated with the development of tolerance, but immunosuppressive techniques based on monoclonal antibodies specific to T cell
molecules are. Monoclonal antibody therapy with αICAM-1 and/or αLFA-1 has led to the development of tolerance in animal models (Benjamin et al., 1988; Charlton et al., 1991; Isobe et al., 1992). If long-term survival of allogeneic myoblasts is due to the development of tolerance, induction of foreign MHC expression by damaged hybrid myofibers should not affect retention of the original myoblasts. Moreover, if haplotype-specific tolerance was induced, a prediction would be that future transplants of the same donor type as the original transplant should be retained but those of a different haplotype will be rejected.

Methods for achieving long-term survival of allogeneic myoblasts in hybrid myofibers without continuous immunosuppression and its associated toxicity are important for the use of myoblast transplantation for therapeutic purposes (Blau et al., 1993; Partridge, 1994). We have shown that transient immunosuppressive treatments lead to the long-term maintenance of allogeneic myoblasts. Elucidation of the mechanisms involved may offer new insights and new directions into better immunosuppressive treatments. Ultimately, it may be possible to engineer a universal donor strain of nonimmunogenic myoblasts deficient in the function (Faustman and Coe, 1991) or expression (Arbonés et al., 1994) of the molecules that mediate myoblast rejection. Transient immunosuppressive techniques coupled with future advances in rendering cells nonimmunogenic should broaden the scope of myoblast-mediated gene therapy for the treatment of disease.

This work was supported by grants from the Dana Fellowship Program in Neuroscience and the Howard Hughes Medical Institute (Physician Research Fellowship) to T. A. Rando and from the National Institutes of Health (HD18179), the Muscular Dystrophy Association, and the March of Dimes Birth Defects Foundation to H. M. Blau.

Received for publication 1 August 1994 and in revised form 29 September 1994.

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