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RAPID COMMUNICATION

# INDUCTION OF SPECIFIC TOLERANCE BY INTRATHYMIC INJECTION OF RECIPIENT MUSCLE CELLS TRANSFECTED WITH DONOR CLASS I MAJOR HISTOCOMPATIBILITY COMPLEX[1]

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**Induction of tolerance to allogeneic MHC antigens has been a goal in the field of transplantation because it would reduce or eliminate the need for generalized immunosuppression. Although encouraging results have been obtained in experimental models by exposing recipient thymus to donor cells before transplantation, donor cells are not typically available at that time, and the donor antigens responsible for the effect are poorly defined. In the present study, thymic tolerance was demonstrated without using donor cells. Recipient thymus was injected before transplantation with autologous myoblasts and myotubes that were genetically modified to express allogeneic donor-type MHC class I antigen. Donor-specific unresponsiveness was induced to a completely MHC-disparate liver transplant and to a subsequent donor-type cardiac allograft, but not a third-party allograft. In vitro, recipient CTL demonstrated a 10-fold reduction in killing of donor cells, but not of third-party cells. Our results demonstrate: (1) that recipient muscle cells can be genetically engineered to induce donor-specific unresponsiveness when given intrathymically, and (2) transfected recipient cells expressing only donor MHC class I antigen can induce tolerance to a fully allogeneic donor.**

Tolerance to self-MHC antigens normally develops via presentation of these antigens on thymic epithelium (*1*). Thymocytes responsive to self-MHC undergo apoptosis resulting in antigen-induced tolerance (*2, 3*). Application of this strategy to tolerance induction in an adult animal transplant model was described by Posselt et al. (*4*), who injected murine islets intrathymically to permit donor-specific unresponsiveness to subsequent islet cell transplants. Their group obtained similar results by injecting bone marrow cells rather than islets into thymus (*5*), suggesting that thymic exposure to donor alloantigen is necessary to promote tolerance. Similar results have been obtained by Goss et al. (*6*) using a rat heart transplant model and intrathymic injection of donor splenocytes. Remuzzi et al. (*7*) prolonged renal transplant survival by intrathymic injection of glomeruli, showing that parenchymal cells can also induce thymic tolerance. Thymic tolerance models in rodents have required thymic exposure to alloantigen near the time of transplantation and concomitant lymphoablative therapy using antilymphocyte serum (ALS)* (*8*) or irradiation (*9*).

In most transplant models of thymic tolerance, thymic exposure to donor cells has occurred several days or weeks before organ transplantation (*4–7*). This approach has limited clinical potential because of the absence of appropriate donor cells before donor organ availability. More importantly, it is not known which donor antigens are necessary or sufficient for tolerance induction. In the present study, we have used a strategy for producing thymic tolerance in a rat model that avoids the requirement of donor cells and allows the study of individual donor antigens using genetically altered recipient cells. We demonstrate the efficacy of intrathymic administration of autologous plasmid-transfected myoblasts (MB) and myotubes (MT) expressing donor MHC class I antigen to induce donor-specific unresponsiveness after liver transplantation in a fully allogeneic, high responder, rat strain combination. Using genetic manipulation, we show a tolerogenic effect with only MHC class I antigen.

## MATERIALS AND METHODS

*Preparation of the primary cell cultures.* Primary muscle cell cultures were prepared as reported previously (*10, 11*). Briefly, minced soleus muscles from baby Lewis rats (3–5 days old) were dissociated in 0.17% trypsin and 0.09% collagenase in HBSS (pH 7.4, Gibco BRL, Gaithersburg, MD) for 55 min in an incubator-shaker at 37°C. After centrifuging, the dissociated cells were resuspended in 3 ml of com-

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* Abbreviations: ALS, antilymphocyte serum; MB, myoblast; MT, myotube; MN4–91–6, murine mAb specific for RT1.A$^a$; pRSVL, plasmid DNA encoding luciferase; pRSVLacZ, plasmid DNA encoding *Escherichia coli* β-galactosidase; pcRT.45, plasmid DNA encoding rat MHC class I RT1.A$^a$; WF, Wistar Furth.

plete media (containing 5% chick embryo extract [Gibco BRL], 15% horse serum, and 80% modified Eagle's media). MB were purified on a Percoll (Sigma Chemical, St. Louis, MO) gradient (20/60×) and $10^6$ cells were placed into individual 35-mm gelatinized culture plates. The cells were then maintained in an incubator (37°C, humidified atmosphere of 5% $CO_2$ in air) with complete media that was changed every other day. After 4 days in culture, the cells spontaneously began to form MT that matured by 8 days in culture.

*Plasmids and gene transfer procedures.* Control plasmids used were plasmid DNA encoding the firefly (*Photinus pyraiis*) luciferase gene (pRSVL) (*12*) and plasmid DNA encoding *Escherichia coli* β-galactosidase (pRSVLac-Z) (*13*), under the control of the Rous sarcoma virus (RSV) long terminal repeat promoter. Plasmid DNA was purified by alkaline lysis, banded 2 times in a cesium chloride-ethidium bromide gradient, dialyzed, and precipitated with ethanol before use.

The cDNA encoding the rat MHC class I antigen RT1.A$^a$ (*14*) was placed in a pcDNA1 plasmid expression vector (pcRT.45) (*15*). The human immediate early CMV promoter and SV40 poly A and intron were used to enhance expression.

Both 2-day-old cultures containing MB and 4-day-old cultures containing MT were transfected as described previously (*10*). Briefly, plasmid DNA and Lipofectin reagent (Gibco BRL) (*16*) were diluted separately into 0.75 ml of Opti-MEM I reduced-serum medium (Gibco BRL). The 2 solutions were then mixed together drop by drop in a polystyrene tube. The DNA-Lipofectin complex solution was added to the primary muscle cells, which had been washed 3 times with Opti-MEM. After a 4-hr incubation, 1 ml of complete medium was added to each plate. Transfection plates were then maintained in the incubator at 37°C, 5% $CO_2$.

*Intrathymic transplantation.* One day after DNA transfection, the MB and MT were harvested using Cell Lifters (Fisher Scientific, Pittsburgh, PA). Five plates of the 3-day-old MB cultures were mixed with 5 plates of the 8-day-old MT cultures in a volume of 0.2 ml containing approximately $10^7$ cells for implantation into 1 recipient animal. The MT formed large rounded cells in suspension that were easily counted using a hemocytometer. Lewis rats were anesthetized by intramuscular ketamine (70 mg/kg) and xylazine (6 mg/kg). A small skin incision was made along the anterior midline at the base of the neck. The upper part of the thymus was exposed by opening the upper sternum in the midline. The genetically modified muscle cells harvested from 10 plates were injected in equal proportions into both lobes of the thymus using a 1.0-ml syringe with a 27-gauge needle. The rat simultaneously received 0.8 ml of ALS (specific for rat lymphocytes; Accurate Chemical & Scientific Corp., Westbury, NY) intraperitoneally. Controls received no ALS.

*Detection of transgene expression in cultured muscle cells.* Flow cytometry was used to detect RT1.A$^a$ expression on pcRT.45-transfected myocytes. Cells were first reacted for 30 min at 4°C with either isotype-control antibody (mouse IgG1, Becton Dickinson, San Jose, CA) or mouse anti-rat RT1.A$^a$ specific antibody (MN4–91–6; Bioproducts for Science, Indianapolis, IN). Cells were washed 2 times with PBS and then were reacted for 30 min at 4°C with goat anti-rat IgG-FITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Cells were washed 2 times with PBS, and were analyzed by flow cytometry.

*Histochemistry.* For evaluation of morphologic changes in transplanted hearts and livers, paraffin sections were stained with Mayer's hematoxylin and eosin.

Cultured MT transfected with pcRT.45 or with control pRSVLacZ were stained immunohistochemically. Cells were fixed with 2% paraformaldehyde in PBS, and were incubated with MN4–91–6 (1:200) overnight at 4°C. A biotinylated goat anti-mouse IgG antibody and avidin-conjugated horseradish peroxidase complex (Vector Laboratories, Inc., Burlingame, CA) were used to reveal MN4–91–6 binding. Substrate was converted to product by 3-amino-9-ethylcarbazole (Sigma).

Immunofluorescent staining was performed on 10-μm cryostat thymus sections. Sections were incubated overnight at 4°C with a mixture of 2 primary antibodies: (1) a rabbit anti-myosin antibody (1:20, Sigma), and (2) MN4–91–6 (1:200) for double-labeling the muscle cells expressing RT1.A$^a$. The sections were incubated with a mixture of fluorescence-conjugated secondary antibodies for 3 hr at room temperature. Myocytes were visualized with goat anti-rabbit IgG-FITC (1:100, Jackson ImmunoResearch) and the RT1.A$^a$ protein was visualized with goat anti-mouse IgG-Texas Red (1:100, Jackson ImmunoResearch).

*Liver transplantation.* Three weeks after thymic transfer of transgenic cells, OLT were performed from male ACI rat donors to male Lewis rat recipients using a technique described previously (*17*). This strain combination was chosen because it normally results in rigorous rejection of the transplanted liver, since the ACI strain is a strong stimulator and the Lewis strain is a strong responder immunologically. The time of rejection was defined by death of the recipient, and rejection was confirmed by histologic examination of the liver. Rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and were cared for according to standard NIH guidelines.

*Limiting dilution analysis.* Twelve replicate cultures containing limiting numbers of Lewis cervical lymph node cells from 3 animals surviving transplant were cocultured with $5\times10^4$ irradiated (2000 rads) ACI or third-party Wistar Furth (WF) splenic stimulator cells per well, as described previously (*18*). After 7 days, $10^4$ $^{51}$Cr-labeled con A-stimulated ACI or third-party splenocytes were added, and $^{51}$Cr release was measured after a 5-hr incubation. Wells with cpm values > 3 SD above the mean spontaneous $^{51}$Cr release were scored as positive. The frequency of CTL precursors was calculated as described previously by Derry and Miller (*19*).

## RESULTS

*Transgenic MHC class I antigen expression.* Approximately 13–30% of primary cultured Lewis rat MB and MT transfected with pcRT.45 (plasmid encoding RT1.A$^a$) tested positive for RT1.A$^a$ surface antigen 48 hr after lipofection, as measured by flow cytometry (Fig. 1), and by inspection of cell cultures using immunohistochemistry (Fig. 2, A and B). One week after intrathymic injection of pcRT.45-transfected mixed MB and MT (MB/MT), the presence of skeletal muscle cells expressing RT1.A$^a$ in the Lewis rat thymus was demonstrated by double-labeling myocytes with antibodies specific for RT1.A$^a$ and myosin (Fig. 3). Myosin-specific staining of the thymus 1 week after implantation confirmed the presence of myocytes containing myosin and myofilaments (Fig. 3, A, C, and E). Myocytes were observed throughout the thymus, and some expressed RT1.A$^a$ (Fig. 3, B, D, and F).

*Intrathymic myocyte transfer and liver allograft survival.* Lewis (RT1.A$^l$B$^l$D$^l$C$^l$) recipients underwent orthotopic ACI (RT1.A$^a$B$^a$D$^a$C$^a$) liver transplants 3 weeks after intrathymic injection of $10^7$ pcRT.45-transfected Lewis MB/MT. Survival times of the recipients were measured with and without ALS treatment (Table 1). The rats receiving no intrathymic injections survived approximately 10–11 days with or without ALS. Thymic injection of MB/MT expressing reporter genes prolonged survival in 5 of 7 recipients treated with ALS, although all 5 recipients eventually died with rejection. Recipients injected intrathymically with pcRT.45-transfected MB/MT, but not receiving ALS, showed a slight prolongation of graft survival to a median of 15 days. Rats receiving pcRT.45-transfected MB/MT intrathymically, as well as ALS intraperitoneally, showed marked prolongation of survival.

Three long-term survivors of the tolerogenic protocol received both ACI and third-party WF (RT1.A$^u$B$^u$D$^u$C$^u$) heart

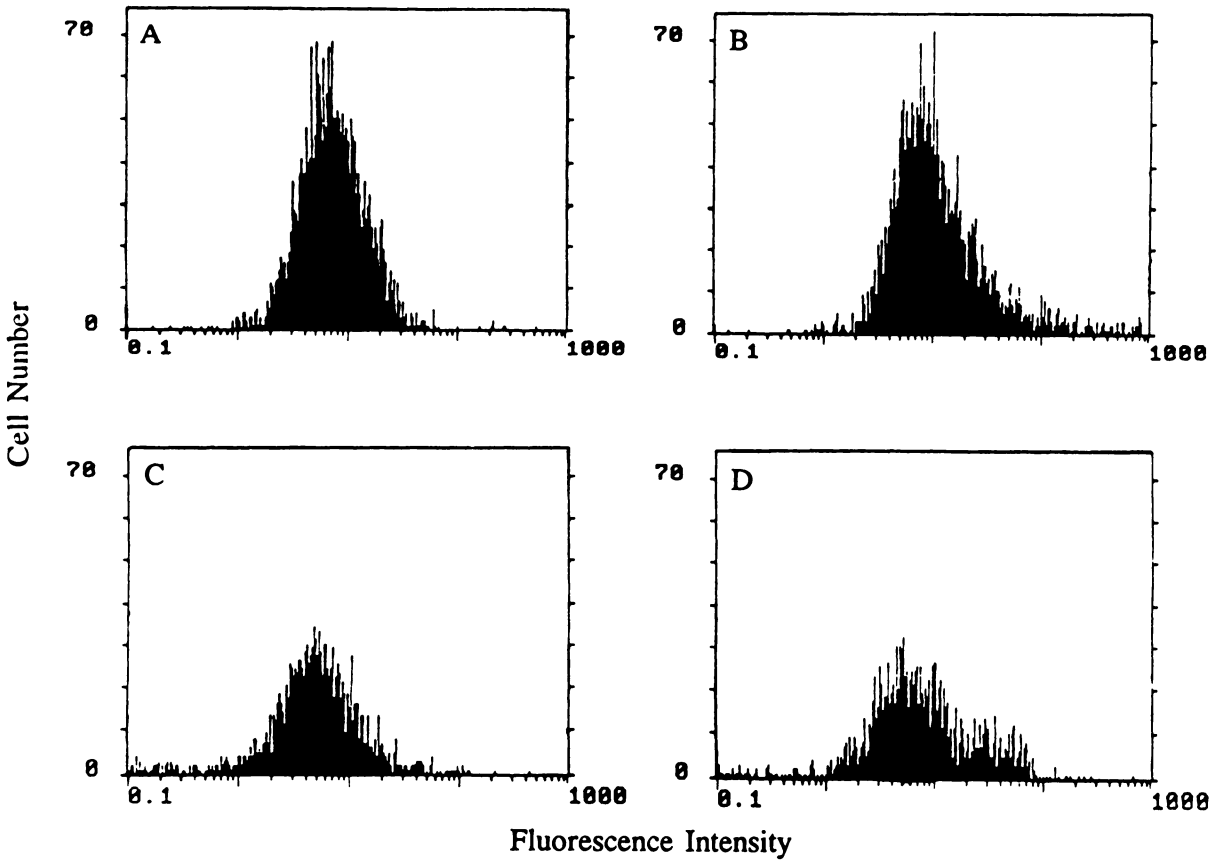


FIGURE 1. Cultured MB and MT transfected with pcRT.45 were analyzed for RT1.A$^a$ expression by flow cytometry after 48 hr. A and C represent transfected MB and MT, respectively, reacted with isotype-control antibody. In B, the same MB were reacted with RT1.A$^a$ antibody (MN4–91–6); D shows the histogram for the MT reacted with MN4–91–6. In the experiment shown, 11% of the MB were positive and MT were 13% positive.

transplants at least 60 days after their liver transplant without any additional immunosuppression. One heart was placed intra-abdominally, and the other anastomosed to the femoral vessels in the groin. In 2 cases, the ACI heart transplants survived indefinitely (> 100 days) without rejection, while the third-party WF hearts were rejected at 8 and 10 days. The third rat died 10 days after heart transplantation for technical reasons, but had rejected the WF heart at 8 days and the ACI heart was still beating just before death.

Histologic examination of multiple sections of ACI livers and hearts transplanted to tolerized Lewis recipients demonstrated no evidence of rejection at the time of death, which was > 100 days after transplantation. Third-party WF hearts underwent acute cellular rejection with a prominent lymphocytic infiltrate seen histologically.

*Cytotoxic T cell frequency analysis.* Compared with the normal Lewis rat response to ACI stimulators and targets, the tolerant Lewis rats demonstrated a markedly diminished CTL precursor frequency for ACI targets (Fig. 4A), yet they maintained a normal CTL precursor frequency against third-party WF targets (Fig. 4B). The mean ± SD frequency of ACI-specific CTL precursors in the 3 experiments with normal Lewis controls was 1/1,995±609, and 1/12,081±3,526 with the tolerized group. The effect was donor-specific, since the CTL precursor frequency against third-party WF targets was 1/3,770±2,045 with Lewis controls, and 1/4,001±1,930 with the tolerized group.

*Routes of transfected myocyte administration.* Controls were used to determine the importance of the site of delivery of the RT1.A$^a$ antigen via transfected MB/MT. Intravenous or intraperitoneal administration of pcRT.45-transfected Lewis MB/MT at the same doses as used for intrathymic injection did not alter graft survival, even with concomitant ALS treatment (Table 2).

## DISCUSSION

Specific tolerance in adults has been achieved in rodent models by intrathymic administration of donor lymphoid cells or parenchymal cells in the setting of ALS treatment (*4–8*). Thymic exposure to allogeneic donor cells permits presentation of alloantigen to maturing thymocytes, mimicking the normal developmental process, and resulting in unresponsiveness or deletion of T cell clones specific for donor alloantigen. Transgenic cells expressing the putative donor MHC antigen necessary for tolerance induction would provide a useful alternative to donor cells. Madsen et al. (*20*) reported the potential usefulness of intravenous administration of cells from a cultured fibroblast cell line transgenically expressing allo-MHC for inducing immunologic unresponsiveness. Recently, donor-type MHC class II allopeptides in-

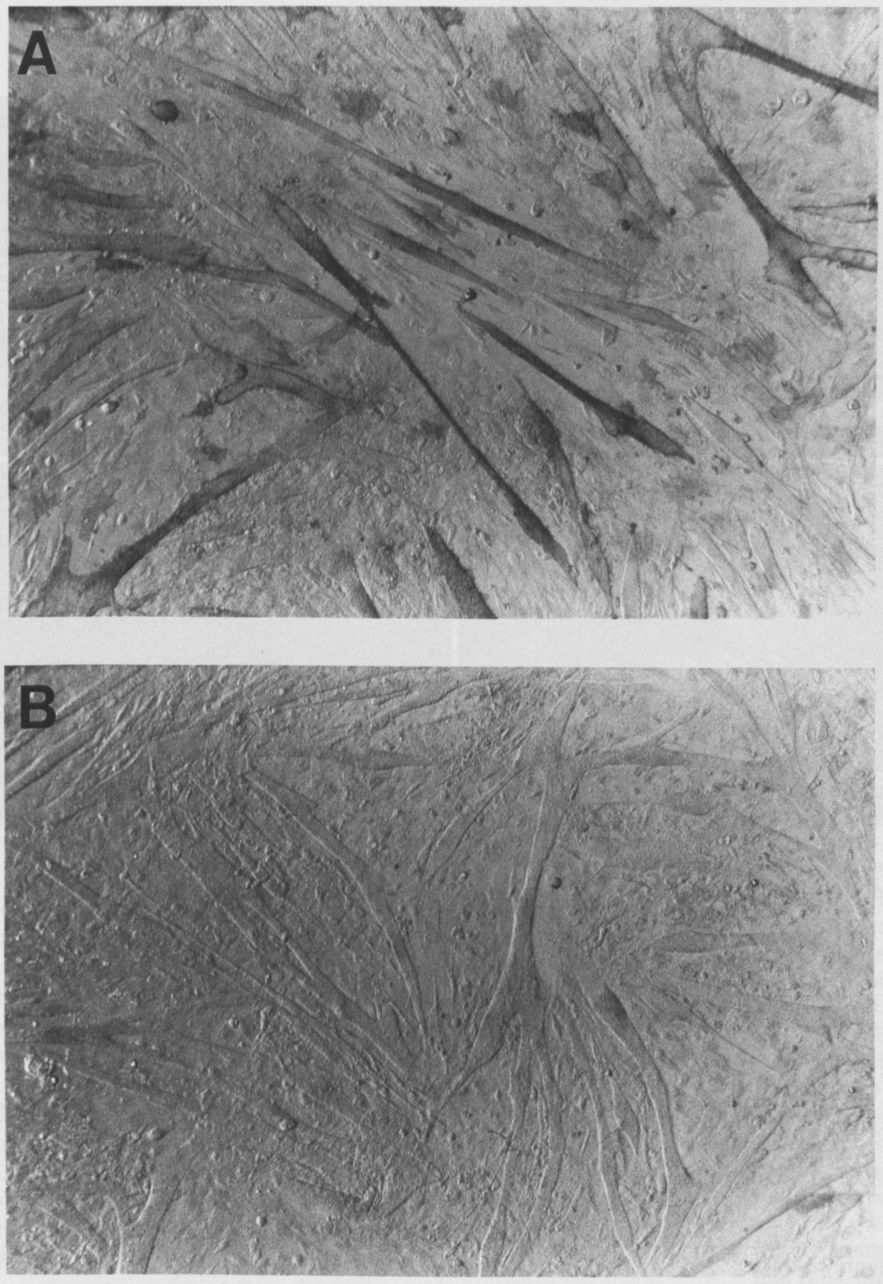


FIGURE 2. Primary cultures of Lewis rat MT; RT1.A$^a$-specific mAb MN4–91–6 stained pcRT.45-transfected MT (A), but not the pRSV-LacZ-transfected control MT (B).

jected intrathymically have also been shown to promote systemic tolerance to subsequent renal allografts (*21*).

In the present study, we achieved tolerance using primary cultured autologous cells expressing only allogeneic MHC class I antigen, and not MHC class II or non-MHC antigens. The results demonstrate that marked prolongation of graft survival, and in some cases donor-specific tolerance, is obtainable with a limited repertoire of donor antigens (only one) in a highly disparate rat strain combination. In fact, the strain combination was chosen specifically because it is a high-responder combination normally resulting in vigorous liver transplant rejection. Tolerance in this model was donor specific, as demonstrated by the results of the in vitro limiting dilution assay showing marked reduction in CTL precursor specific for ACI donor cells. Furthermore, the subsequent acceptance of the tolerized donor-strain heart transplant and rejection of the third-party donor heart transplant demonstrates in vivo evidence of donor-specific tolerance. The in vitro results were consistent with these, suggesting that the mechanism of thymic unresponsiveness in this model is due at least in part to a decrease in CTL responsiveness to the donor.

It is notable that intrathymic injection of MB/MT expressing only the reporter gene prolonged survival in a few animals. This effect might have been due to the nonspecific influence of thymic injection, since Staples et al. (*9*) demonstrated that injection of bovine γ-globulin into rat thymus results in depressed immunity to subsequent skin grafts.

The role of the thymus appears to be central. Transgenic cells administered intravenously or intraperitoneally were not effective at inducing tolerance, demonstrating the superiority of the thymic route. The form of the antigen expressed

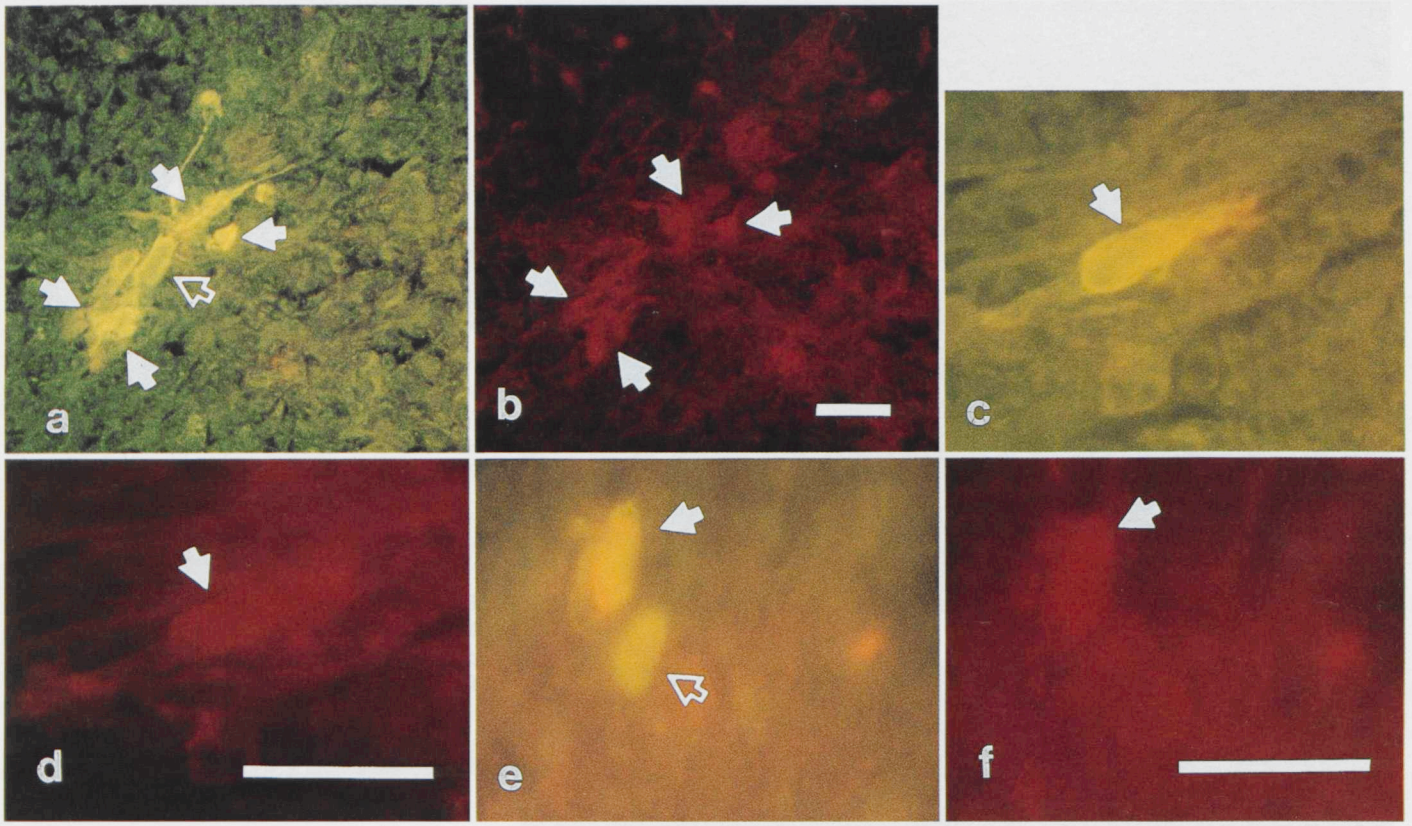


FIGURE 3. Micrographs of the thymus sections using double immunofluorescence show implanted myocytes (a, c, and e) and myocytes expressing RT1.A[a] (b, d, and f). Closed arrows indicate double-labeled muscle cells; open arrows indicate muscle cells labeled only with anti-myosin. Scale bars = 30 μm.

TABLE 1. Survival of ACI to Lewis liver transplant recipients (days)

| Group | Intrathymic injection | Survival (days) (mean ± SD) | | $P^a$ |
|---|---|---|---|---|
| | | -ALS | +ALS | |
| 1 | None | 9, 10, 10, 10, 11 (10±0.7) | 10, 11, 11, 14 (11.5±1.7) | 0.13 vs. 1– |
| 2 | MB/MT with pRSVLux or pRSVLacZ | 9, 10, 12, 14 (11.3±2.2) | 10, 12, 18, 23, 24, 25, 75 (26.7±22) | 0.02 vs. 2–<br>0.03 vs. 1+ |
| 3 | MB/MT with pcRT.45 | 13, 15, 15, 18 (15.3±2.1) | 13, 14, 18, 18, 44, 56, 72, 74, (76),[b] 80, 91, >100, 121, (164),[b] (304)[b] (83±75) | 0.003 vs. 3–<br>0.02 vs. 2+ |

[a] Log rank survival analysis compared by chi-square tests.
[b] Parentheses indicate the rat was killed.

by transgenic cells may also be a factor. Behara et al. (*22*) have reported that thymic implantation of genetically modified fibroblasts do not lead to tolerization toward a soluble gene product (growth hormone). Failure to induce tolerance after thymic exposure to genetically modified fibroblasts may have been related to the level of gene expression in the thymus or the soluble nature of the gene product, since both of these parameters influence immune responsiveness.

The role of the liver transplant in testing tolerance induction with MHC class I antigen in this model may be substantial for 3 reasons. First, unresponsiveness to liver transplants is usually easier to achieve than unresponsiveness to either cardiac or renal transplants. For example, rat liver transplants can be substantially prolonged by intravenously administering blood cells expressing donor MHC class I antigen, whereas cardiac allograft survival is not prolonged by the same protocol (*23*). Second, MHC class I antigen may be more important for tolerance induction in liver transplant models (*24*) than in other organ models. Third, the acceptance of ACI cardiac allografts after ACI liver transplantation may be due in part to a tolerogenic effect of the transplanted liver. This phenomenon has been reported in a low-responder strain combination (*25*), although the mechanism is unknown.

Gene transfer to autologous cells provides a method to study the role of specific gene products in thymic tolerance without the interference of other donor antigens. Specific immunologic unresponsiveness to a fully MHC-disparate liver following presentation of only donor MHC class I in the thymus suggests that if manipulation of the recipient immune response favors unresponsiveness rather than rejection of one important target antigen, other existing MHC and

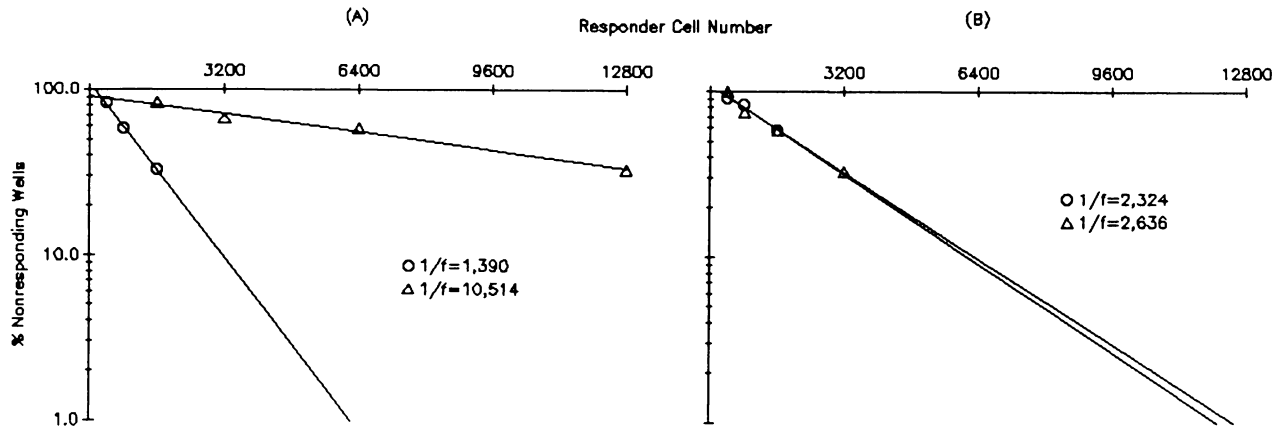


FIGURE 4. CTL precursor frequency was assessed in Lewis recipients of ACI liver allografts pretreated with ALS and intrathymic Lewis myocytes expressing RT1.A$^a$. Experiment shown is representative of three. Cervical lymph node CTL precursor frequency was assessed in a normal Lewis control (open circles, dashed lines), and an ALS/myocyte-pretreated Lewis recipient of an ACI liver allograft 60 days after transplant (open triangles, solid lines) using (A) ACI or (B) third-party WF splenocyte stimulators and targets. The CTL precursor frequency for respective targets is shown on each graph.

TABLE 2. Survival of ACI to Lewis liver transplant recipients 3 weeks after transgene administration intravenously or intraperitoneally with ALS 0.8 ml i.p.

| Route | Survival (days) |
|---|---|
| Intraperitoneal injection MB/MT with pcRT.45 | 9, 10, 12, 14 |
| Intravenous injection MB/MT with pcRT.45 | 10, 11, 13, 17 |

non-MHC disparities may be ignored by the recipient immune system and will not necessarily eventuate in rejection. A biological approach to tolerance may therefore be feasible if the most important, rather than all, of the antigenic disparities between donor and recipient are addressed. Which antigens are most important may vary according to the organ transplanted, with our data suggesting an important role of MHC class I in liver transplantation, consistent with results in clinical transplantation (*26*). The important role of MHC class II antigens in renal transplant rejection (*27*) is paralleled by the finding that thymic presentation of donor MHC class II peptides prolongs subsequent renal allografts in rats (*21*).

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