

## Successful Islet Allotransplantation in Diabetic Rats Immunosuppressed With FK506: A Functional and Immunological Study

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The effect of a novel immunosuppressive agent, FK506, on fresh islet allografts was evaluated in diabetic rats across major histocompatibility complex (MHC) barriers with respect to the transplantation (TR) site, islet source, treatment regimen, and antidonor antibody (Ab) titers of the recipients after TR. The functional periods of Wistar (Wi) islets transplanted under kidney capsule (KC) or intraportally (IPo) and of a mixture of Wi and Lewis (Le) islets under KC or IPo in nonimmunosuppressed ACI rat recipients were  $6.9 \pm 0.4$  (n = 7),  $6.4 \pm 0.5$  (n = 7),  $5.8 \pm 0.4$  (n = 7), and  $6.2 \pm 0.4$  (n = 5) days, respectively. FK506 treatment at 1 mg/kg/d intramuscularly (IM) for 2 weeks (protocol I) following islet TR under KC and IPo significantly prolonged the allograft function to more than  $71.8 \pm 11.3$  (n = 10) and  $161.7 \pm 18.6$  (n = 11) days, respectively. Additional treatment with FK506 at 1 mg/kg/wk (protocol II) further increased the islet survival under KC to more than  $212.6 \pm 22.3$  (n = 8) days. With this FK506 treatment protocol, the Wi + Le mixed-islet allograft function was extended to more than  $106.1 \pm 10.5$  (n = 7) and  $167.9 \pm 28.6$  (n = 7) days under KC and IPo, respectively. Nephrectomy in 8/8 ACI rats with long-term-functioning Wi (n = 6) and Wi + Le (n = 2) islet allografts resulted in their return to hyperglycemia. Immunohistochemical staining showed abundant insulin-positive cells at the graft site, with small numbers of CD4- and CD8-positive cells present in the vicinity of the normal-appearing islets. Macrophages were not detected. The immunosuppressive effect of FK506 was further tested in ACI rats pre-sensitized by a previous Wi islet TR. When the duration between the first and second TR under KC was  $114.3 \pm 20.5$  days, protocol II treatment significantly prolonged the graft function to more than  $152.9 \pm 28.7$  (n = 8) days. However, with a short duration of about 2 weeks between the two TRs, the same FK506 protocol achieved islet graft function of  $14.0 \pm 3.8$  days (n = 7). Additional immunosuppression with cyclophosphamide did not further improve the survival time. Antidonor Abs detected in ACI recipients of Wi islet allografts were significantly lower in the FK506-treated animals compared with the nontreatment group. Wi and Le skin grafts performed in three ACI rats with long-term-functioning Wi islets IPo caused the rejection of the islet allografts. Skin grafts were also rejected in the first-set fashion. Six ACI recipients with long-term-functioning IPo Wi islet allografts were rendered hyperglycemic by streptozocin (STZ) injection. Long-term normoglycemia without further FK506 immunosuppression was achieved following retransplantation with fresh Wi islets IPo (n = 2), but not under KC (n = 2). The results of the present study indicate that FK506 was an effective immunosuppressant for islet allotransplantation in diabetic ACI rats across MHC barriers with islets from two donor strains, as well as in sensitized recipients whose antidonor activities had subsided. The efficacy of the immunosuppression was influenced by the FK506 treatment protocol and the site of the islet transplant. The results suggest that FK506 could be useful in clinical islet TR.

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**I**SLET TRANSPLANTATION (TR) has been shown to restore normoglycemia and prevent the development of chronic complications in diabetic animals.<sup>1,2</sup> The application of allotransplantation and xenotransplantation of pancreatic islets for the treatment of diabetes is hindered by immune rejection. FK506, a new immunosuppressant, has been demonstrated to be many times more potent than cyclosporin A in the suppression of mixed leukocyte reaction *in vitro*.<sup>3</sup> We have earlier shown that FK506 was an effective immunosuppressive agent for fresh islet allograft across the major histocompatibility complex (MHC) barrier.<sup>4</sup> The efficacy of FK506 in the prolongation of islet allograft survival has been found to be influenced by the dosage of FK506 and the site of the islet graft.<sup>4,5</sup> The present study was undertaken to determine whether FK506 was effective in the prolongation of fresh islet allograft in sensitized diabetic rat recipients and in recipients of islets from one or two donor strains in two commonly used TR sites (kidney capsule [KC] and intraportal [IPo]). The immunologic status, including the possibility of tolerance induction, in recipients with long-term islet allograft function was also investigated.

### MATERIALS AND METHODS

#### Animals

Male rats of outbred Wistar (Wi) and inbred Lewis (Le) strains (RT1<sup>b</sup>) with body weights of 350 to 500 g were used as donors of

pancreatic tissue, and rats of inbred ACI (RT1<sup>a</sup>) strain were used as streptozocin (STZ)-induced (55 mg/kg IV) diabetic recipients (Harlan-Sprague-Dawley, Indianapolis, IN). An animal was defined as diabetic only when the serum glucose level was greater than 400 mg/dL for more than 10 days.

#### Islet Isolation and TR

Pancreatic tissue was digested with collagenase, and the islets were hand-picked under a dissection microscope. Contaminating acinar tissues and blood vessels were removed from the islets by the single-layer Hypaque-Ficoll (H-F) separation technique.<sup>6</sup> For KC TR, approximately 2,000 freshly isolated islets suspended in a total volume of 70  $\mu$ L Hanks balanced salt solution (HBSS) were injected. For IPo TR, the islets were suspended in 200  $\mu$ L HBSS in a Monoject U100 insulin syringe (Sherwood Medical, St Louis, MO) and injected over a 1-minute period into diabetic recipients. The syringe was flushed twice with the recipients' blood.

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### Immunosuppression Protocol

The injectable form of FK506 (lot no. 116393) was provided by Fujisawa Pharmaceutical (Osaka, Japan). The required amount of the compound was weighed out daily and prepared in saline within 10 minutes of intramuscular (IM) injection. The administration of FK506 was initiated on the day of TR. Protocol I consisted of FK506 at 1 mg/kg/d IM for 2 weeks. Protocol II consisted of FK506 IM administration at 1 mg/kg/d for 2 weeks followed by 1 mg/kg/wk. Protocol III consisted of FK506 IM administration at 1 mg/kg/d for 2 weeks followed by 1 mg/kg/wk plus cyclophosphamide at 20 mg/kg/d on days -1, 0, and +1.

### Assessment of Graft Function

Daily serum glucose levels and body weights of recipient rats were determined for 2 weeks after TR and then twice weekly thereafter. Rejection was considered to have occurred when the serum glucose level exceeded 200 mg/dL on 3 successive days. Survival time for each recipient group is reported as the mean  $\pm$  standard error of the mean. Statistical evaluation was performed by the Mann-Whitney *U* test. *P* value of less than .05 was considered statistically significant.

An intravenous glucose tolerance test (IVGTT), 1 g/kg IV through the femoral vein in overnight-fasted animals, was performed in some islet recipients 1 week after the last dose of FK506. Blood samples were collected from the tail at 0, 1, 3, 5, 15, 30, 60, 90, and 120 minutes for glucose determination.

### Assessment of the Efficacy of FK506 Immunosuppression on Islet Allograft Survival in Sensitized Recipients

Diabetic ACI rats were transplanted with fresh Wi islets under the left KC. After rejection of the first islet allograft, a second islet graft from the same donor strain was performed in the contralateral kidney of the recipient. The second TR was performed either a short or long period after the first TR. The animals were treated with FK506 as indicated.

### Antibody Studies

Collected sera were stored at  $-20^{\circ}\text{C}$  and heated at  $56^{\circ}\text{C}$  for 30 minutes before antibody (Ab) determination. Cytotoxic Ab levels in the ACI recipients of fresh Wi islets with and without FK506 treatment were determined using pooled Wi strain splenocytes as target cells and rabbit serum as the complement source (Low-Tox R complement, Cedarlane Laboratory, Hornby, Ontario, Canada).

Ab titer is defined as the reciprocal of serum dilution that kills 50% of the target cells by trypan blue dye exclusion assay.<sup>7</sup>

### Assessment of Tolerance

To assess the possibility of tolerance in rats with long-term-functioning Wi islet allografts, Wi and Le skin grafts were implanted in the lateral thoracic region of the ACI recipient. Survival of the islet and skin grafts were monitored. In addition, six rats with long-term-functioning islet allografts were rendered diabetic with STZ at 35 mg/kg. After 10 days of hyperglycemia, retransplantation of fresh Wi islets IPo or under KC was performed in these rats. The recipients did not receive any further FK506 treatment. Serum glucose level was monitored as an indicator of islet allograft function.

### Immunohistochemical Studies

Some functional grafts were removed for immunohistochemical stainings. Paraffin sections were stained for insulin and glucagon with immunoperoxidase staining (ABC Staining Kits, Vectastain, Dimension Laboratory, Mississauga, Ontario, Canada). Frozen sections were stained with monoclonal Abs for leukocyte common antigens, class II antigens, CD8 and CD4 lymphocyte subsets, and macrophages (Cedarlane Laboratory).

### RESULTS

Table 1 shows the functional period of Wi and admixed Wi and Le islet allograft survival in two TR sites in diabetic ACI rats treated with two different FK506 protocols. FK506 protocol I treatment significantly prolonged the survival of islet allograft transplanted both under KC and IPo. The survival was significantly longer in the IPo site than in KC (group 7 v 5, 11 of 11 v three of 10 functioned for > 110 days). In the KC site, the islet survival for recipients treated with FK506 for 2 weeks was more than  $71.8 \pm 11.3$  days (group 5), and this increased significantly to more than  $212.6 \pm 22.3$  days (group 6) when an additional weekly injection of FK506 was given. In the groups transplanted with a mixture of Wi and Le islets, FK506 treatment significantly improved the islet allograft functional period over that of the untreated recipients (group 8 v 3). Also, the group transplanted IPo had a longer functional period of the mixed Wi and Le allografts than the group transplanted

Table 1. Functional Period of Wi and Admixture of Wi and Le Islet Allografts in Diabetic ACI Rats Immunosuppressed With FK506

Group	Donor	Site	FK506 Treatment†	n	Days	Mean $\pm$ SEM
1	Wi	KC	No	7	5,6,7,7,8,8	6.9 $\pm$ 0.40
2	Wi	IPo	No	7	5,5,6,6,7,8,8	6.4 $\pm$ 0.48
3	Wi + Le	KC	No	7	4,5,5,5,6,7,7	5.6 $\pm$ 0.43
4	Wi Le	IPo	No	5	5,6,6,7,7	6.2 $\pm$ 0.37
5	Wi	KC	I	10	41,45,46,47,48,52,73, > 120, > 122*, > 124*	> 71.8 $\pm$ 11.3
6	Wi	KC	II	8	> 112*, > 114*, > 212*, > 245, > 251, > 252, > 255, > 260*	> 212.6 $\pm$ 22.3
7	Wi	IPo	I	11	2 x 110, 4 x > 122, > 167*, > 191*, > 193*, > 210, > 310‡	> 161.7 $\pm$ 18.6
8	Wi + Le	KC	II	7	67,85,90*, 103,120*, 132, > 145	> 106.0 $\pm$ 10.5
9	Wi + Le	IPo	II	7	98, 102, 110, 120, 240, > 252, > 253	> 167.9 $\pm$ 28.6

\*Nephrectomy.

†Protocol I, FK506 at 1 mg/kg/d for 2 weeks IM; protocol II, FK506 at 1 mg/kg/d for 2 weeks + weekly IM.

‡Skin graft.

§STZ, 35 mg/kg IV.

¶Died of anesthesia.

under the KC (group 8 v 9). However, FK506 efficacy was lower for mixed islet allograft than for islets from a single donor strain in the KC but not IPo (group 6 v 8,  $P < .01$ ; 7 v 9, NS).

Glucose metabolism was improved in ACI rats with functioning Wi islets. The glucose clearance (K) rates of IVGTTs performed in ACI recipients with functional Wi islets under KC 1 week after the last injection of FK506 were  $2.16 \pm 0.09$  (group 5,  $n = 6$ ) and  $1.83 \pm 0.06$  (group 6,  $n = 7$ ), which were much improved over rates of the diabetic controls (K rate  $< 1.0$ ) but significantly lower than those of normal control animals ( $K = 3.10 \pm 0.11$ ,  $n = 11$ ).

To confirm that the long-term normalization of blood glucose levels in ACI rats with Wi islets was not due to spontaneous reversion of diabetes, graft removal was performed in some animals. Nephrectomy of the graft-containing kidney resulted in the return to hyperglycemia in six out of six cases of ACI rats with Wi and two out of two with Wi + Le islet allografts. Wi islet allograft with normal-appearing histology was observed under the KC 114 days after TR in diabetic ACI rats immunosuppressed with protocol II. Insulin-containing islets were abundant. In some areas of the graft, small numbers of CD4-positive and CD8-positive cells were more prevalent. Staining for macrophage with monoclonal clones OX41 and OX42 and also with nonspecific esterase staining failed to detect them at the graft site. Normal-appearing islets were also present in the graft area from diabetic recipient rats subjected to FK506 protocol I. However, more aggregates of CD4-positive and CD8-positive cells were present adjacent to the islets than observed in the previous group.

Figure 1 shows the serum glucose levels in six ACI

recipient rats with long-term-functional Wi IPo allograft (Table 1, group 7) reinduced to become diabetic by STZ. Retransplantation of two of these animals with fresh Wi islets IPo resulted in prolonged normoglycemia of the recipients without further FK506 immunosuppression. One rat died of surgical complications 30 days after retransplantation, with functional islet allograft at death. In the second animal, a Wi skin graft performed at day 90 resulted in its return to hyperglycemia in 9 days. In contrast, the two animals that received the retransplantation of fresh islet allograft under the KC rejected the grafts in 5 and 8 days. Two rats without retransplantation remained hyperglycemic.

The Wi rat islets that functioned long-term IPo in diabetic ACI rats still retained their antigenicity. Wi strain skin graft performed in these animals induced islet graft rejection in three out of three cases between 9 and 13 days. Both Wi and the third-party Le skin grafts were rejected in the first-set fashion in 12 to 13 days.

Table 2 shows the efficacy of FK506 protocol II treatment on islet allograft survival in diabetic ACI recipients that had rejected a Wi islet graft performed 50 to 177 days previously. The immunosuppression was effective in achieving long-term survival in all eight recipients, with three out of eight recipients achieving indefinite graft survival. This was not significantly different from that achieved in nonsensitized animals (Table 2 group 3 v Table 1 group 6,  $P = .10$ ). In contrast, when the interval between the first and second TR was of shorter duration, ie, 2 weeks, FK506 immunosuppression alone (group 4) and in combination with cyclophosphamide (group 5) was found to be ineffective, with only

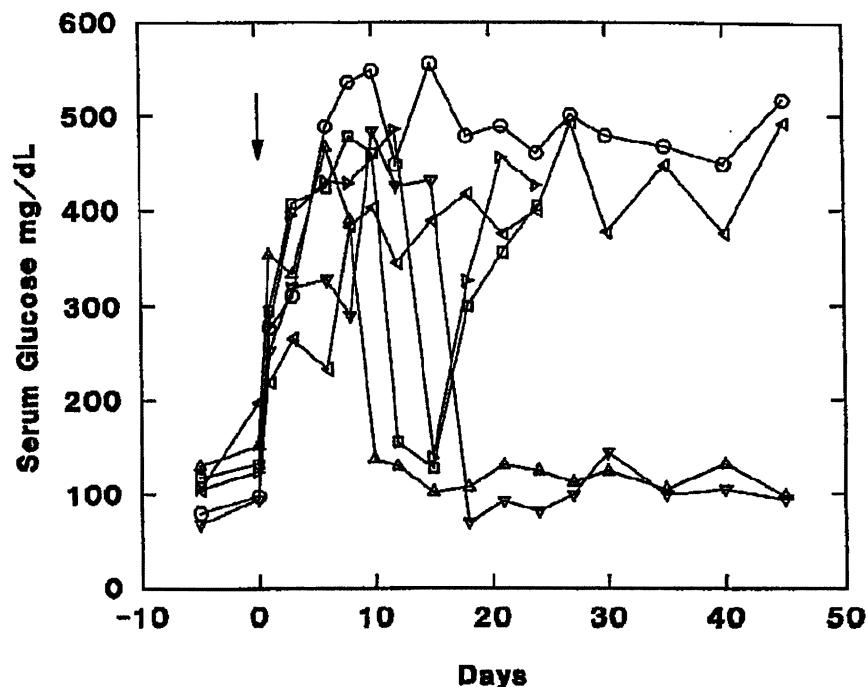


Fig 1. Serum glucose levels of ACI rats with long-term-functional IPo Wi islet allografts rendered diabetic by STZ injection (arrow) and then either retransplanted with fresh Wi islets IPo ( $\nabla$ ,  $\Delta$ ) or under KC ( $\square$ ,  $\diamond$ ), or without retransplantation ( $\circ$ ,  $\times$ ).

Table 2. Effect of FK506 Treatment on Functional Islet Allograft Survival Under KC in Pre-sensitized ACI Rats

Group	Donor	Days 1st-2nd Tr	FK506 Treatment*	n	Days	Mean $\pm$ SEM
1	WI	No	No	6	5,5,6,6,7,7	6.0 $\pm$ 0.37
2	WI	106.3 $\pm$ 4.5	No	6	6,7,7,7,8,8	7.17 $\pm$ 0.31
3	WI	114.3 $\pm$ 20.5	II	8	59,78,99,113, > 145,201, > 258, > 270	> 152.9 $\pm$ 28.7
4	WI	15.3 $\pm$ 0.3	II	7	5,5,10,11,14,19,24	14.0 $\pm$ 3.8
5	WI	14.20 $\pm$ 0.5	III	5	8, 10,16,19,21	14.8 $\pm$ 2.5

NOTE. Sensitization was achieved by fresh WI islet allograft under KC.

\*Protocol II, FK506 at mg/kg/d for 2 weeks + weekly IM; protocol III, FK506 at 1 mg/kg/d for 2 weeks + weekly IM; cyclophosphamide 1 mg/kg/d.

one recipient having a prolonged functional graft. Nevertheless, hyperacute rejection was not observed.

Figure 2 shows that in ACI rats without FK506 immunosuppression, anti-islet donor Ab titers increased rapidly and peaked at day 14 at  $77.3 \pm 0.9$  ( $n = 12$ , mean  $\pm$  SEM). In comparison, the anti-WI Ab titers were significantly lower in islet allograft recipients treated with FK506, with the peak titer at  $11.1 \pm 2.4$  ( $n = 7$ ) on day 7. The anti-WI Ab was negligible in the recipients at the time of the second TR when the interval between the first and second TR was more than 50 days in sensitized recipients. The peak Ab titers remained low at  $8.0 \pm 2.2$  ( $n = 5$ ) after the TR while immunosuppressed with FK506. In comparison, in the recipients that had recently rejected the first WI islet allograft, anti-WI Ab titers were high, with a mean of  $115 \pm 12.8$  ( $n = 5$ ), and increased further to 512 on day 8 after the second TR despite FK506 immunosuppression.

#### DISCUSSION

Results of the present study show that FK506 was an effective immunosuppressant in prolonging fresh islet allograft survival across the MHC barrier. At a daily dosage of 1 mg/kg administered IM for 14 days starting on the day of TR, significant prolongation of fresh WI islet allograft survival was observed both under the KC and IPO. The

efficacy of FK506 treatment was improved by additional weekly treatment in the group with islets transplanted under KC. This treatment regimen was also effective in prolonging the survival of islet allograft composed of tissues from two donor strains. The observation may be of some importance, since most studies show that islet tissue from more than one donor is needed to reverse the diabetic state of the recipients in clinical TR, despite a recent report showing successful clinical islet allotransplantation with islets procured from a single donor.<sup>8-10</sup> Although FK506 prolonged islet allograft survival from single- and two-donor strains transplanted under KC, the result achieved in the former group was significantly better. One possible explanation is the higher immunogenicity of the mixed-islet preparation.

Earlier, Yasunami et al<sup>5</sup> failed to achieve prolonged islet allograft survival under KC. This difference could be due to the lower dosage of FK506 administered for a shorter period than in the present study. The route of TR seems to be important, since we observed the superiority of the IPO over the KC site in the islet TR model. This confirms previous observations.<sup>5</sup> The suggestion that FK506 is metabolized extensively in the liver before excretion may contribute to the superiority of the IPO site.<sup>11</sup>

Despite voluminous literature on the effect of various immunosuppressive agents and islet pretreatment protocols on islet survival in nonsensitized animals, relatively little information is available for sensitized animals. The data demonstrated that FK506 was effective in prolonging the second islet allograft survival in sensitized rats, provided the antidonor immune activities had subsided.

Our results would indicate that the classic form of tolerance was absent in rats with long-term islet allograft function, as they failed to retain the donor-strain skin graft. Furthermore, the retransplanted islets were susceptible to induction of rejection by donor-strain skin graft. However, some form of tolerance was present in these animals, since retransplantation of fresh donor-strain islets IPO following the destruction of the islet graft with STZ resulted in prolonged normoglycemia without further FK506 immunosuppression. This observation is therefore similar to that of Kamei and Yasunami, who observed a similar tolerance state induced in their rats with cyclosporin A treatment.<sup>12</sup>

Ricordi et al<sup>13</sup> have provided experimental evidence that the function of human islets residing underneath the KC of nude mice is deleteriously affected by the administration of FK506 at doses of 1 mg/kg/d and higher. In addition,

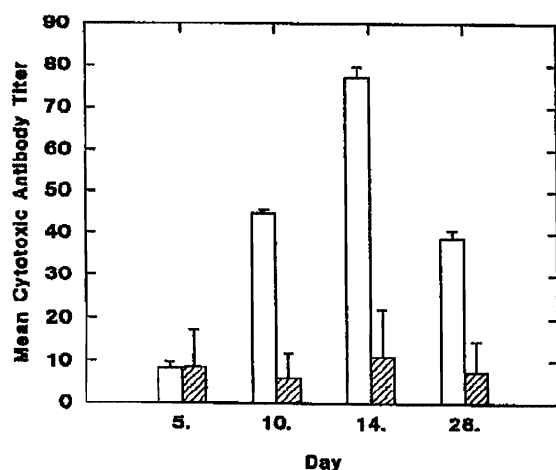


Fig 2. Mean cytotoxic Ab titers in ACI recipients of fresh WI islet allograft under KC with (■, mean  $\pm$  SEM,  $n = 7$ ) and without (□,  $n = 12$ ) FK506 treatment.

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FK506 has been shown to be diabetogenic in intact nontransplanted animals.<sup>14,15</sup> This effect on carbohydrate metabolism is reversible following the discontinuation or dose reduction of the medication. Furthermore, FK506 has been used in clinical islet allotransplantation with mixed results.<sup>16</sup>

In conclusion, FK506 is a potent, effective immunosuppressant for islet allograft TR in diabetic rats across the MHC barrier. The immunosuppressive effect was observed in recipients of islets from more than one donor strain and in presensitized recipients. In the sensitized recipients, the

long interval between the initial TR and retransplantation was crucial to the survival of the second transplant using FK506 as the antirejection agent. The results of this study would provide useful information for clinical islet TR. Improvement of the treatment regimen using FK506 should minimize the side effects, and allow it to become useful in clinical islet TR.

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