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Long-Term Insulin Independence Following Repeated Islet Transplantation in Totally Pancreatectomized Diabetic Pigs

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Clinical islet transplantation (Tx) in type I diabetic patients has been successful so far only in a minority of cases, probably because of multiple factors, partly immunologic and partly nonimmunologic in nature. Preclinical studies of islet Tx in large animals are still needed to clarify the reasons and find possible solutions. In this study, we tested the feasibility of noninvasive, repeated intrahepatic allo-Tx of porcine pancreatic islets obtained from multiple donors, in pigs rendered diabetic by total pancreatectomy (Pct). In group I Yucatan miniature swine ($n = 6$), after induction of diabetes by Pct, repeated islet allo-Tx of ≥80% pure islets was performed. Islets obtained from two pigs of the Hanford breed were injected twice a week, half freshly isolated and half 48-h cultured, over a period of 11 days, for a total of 23,647 ± 1617 islet equivalents (IE)/kg recipient body weight (BW). In group II Yucatan miniature swine ($n = 3$), after Pct, a single allo-Tx of ≥80% pure islets, previously obtained from two donors of the Hanford breed, was performed, using a total of 22,416 ± 1124 IE/kg BW. In group III Yucatan miniature swine ($n = 3$), auto-Tx of 60–75% pure islets, averaging 2980 ± 424 IE/kg BW, was performed a few hours after Pct. Group IV Yucatan mini pigs ($n = 3$) underwent Pct and were used as diabetic controls. Group V animals ($n = 3$) were normal control Yucatan mini pigs. Porcine islets were isolated by a modification of the standard collagenase digestion and Ficoll gradient purification method. Donors and recipients were chosen on the basis of moderate to high mutual alloreactivity in mixed lymphocyte culture (MLC). In groups I and II, cyclosporine A (CsA) was started 4 days before allo-Tx, at the dose of 15 mg/kg IM, and then gradually reduced to 4 mg/kg IM. In all group I animals, normal fasting blood glucose (FBG) was restored within 2–3 weeks. Two normoglycemic pigs died of acute pneumonia at 33 and 112 days, respectively, and one animal became progressively hyperglycemic at 100 days. After 3 months, discontinuation of CsA treatment resulted in FBG increase in two group I animals. In one pig, CsA was stopped after 151 days, and normoglycemia persisted until euthanasia, after 8 months. In group II pigs, normoglycemia lasted 4–20 days, with a progressive increase of insulin requirement thereafter. In group III animals, after islet auto-Tx, normoglycemia lasted 7–10 days, while insulin daily requirement progressively increased thereafter, stabilizing at 0.4 IU/kg/day, corresponding to about one third of the amount required in diabetic controls. The single most important result in this series of experiments is that intraportal allo-Tx of a sufficient islet mass, divided in multiple subtherapeutic doses, produced a better metabolic long-term control in comparison to a single injection of the same amount of islets. The technique of multiple-donor repeated islet Tx may prove useful to overcome the problem of primary nonfunction or early graft failure, currently limiting the success of clinical islet Tx in most cases.

Key words: Repeated islet transplantation; Experimental diabetes; Total pancreatectomy

INTRODUCTION

Tight glucose control through intensive insulin treatment is associated with less severe or delayed development of chronic complications of type I diabetes, but is often fraught with multiple episodes of severe hypoglycemia (7). On the other hand, transplantation (Tx) of islets of Langerhans, when successful, is characterized by excellent metabolic control in the absence of hypoglycemia (6). However, long-term insulin independence after islet allo-Tx is reached only in a minority of cases (12). The reasons for limited success are probably multifactorial, and include both immunologic problems, such as rejection and recurrence of autoimmunity, and nonimmunologic causes, like insufficient functional β cell mass, poor engraftment, incompatibility between human blood

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and isolated islets, and direct toxicity on β cells by immunosuppressive drugs (3,6,12,31).

Islet allo-Tx in pigs shares with human islet allo-Tx the problem in isolation of a sufficient islet mass from a single pancreas and the difficulty in controlling immunologic rejection. For these reasons, long-term success in pigs has not been consistently achieved so far (21), and normoglycemia lasting more than 1 month was reported only with the use of a strong multiple-drug immunosuppressive regimen (22). We previously showed that repeated intraportal injections of subtherapeutic islet cell isografts restore normoglycemia in streptozotocin-diabetic rats (23). Similar results were also obtained on pancreatectomized diabetic pigs, as previously published in a preliminary report (24). This effect is consistent with a key role of engraftment for the success of islet Tx. We report herein the ultimate results of a series of experiments in which we performed repeated islet Tx in pigs rendered diabetic by total pancreatectomy (Pct), under sole cyclosporine (CsA) treatment. The effect of multiple intraportal islet injections was compared with that obtained by allo-Tx of the same amount of islets in a single infusion, as well as to islet auto-Tx.

MATERIALS AND METHODS

Animals

Islet donors were outbred, 1-year-old, female Hanford mini pigs, weighing 55–65 kg (Charles River, Wilmington, MA). Recipients were female, 2–3-month-old, Yucatan mini pigs, averaging 9–13 kg (S&S Farms, Ranchita, CA). For auto-Tx experiments, 1-year-old Yucatans, 50–60 kg, were used (S&S Farms, Ranchita, CA). Animals were pair-housed and fed twice daily by a standard commercial chow (Purina Mills Inc., St. Louis, MO). All experiments were performed in accordance with the National Research Council's criteria for humane care, after local Institutional Animal Care and Use Committee approval. Animals were clinically assessed daily, and were weighed twice a week. After Pct, blood glucose was checked twice/three times a day by an enzymatic colorimetric method (One Touch II Meter and Test strips; Lifescan Inc., Milpitas, CA). Blood samples were obtained at weekly intervals for routine laboratory tests and plasma lipoproteins. After Pct, short-duration insulin (Regular Purified Pork Insulin Injection USP, Novo Nordisk Pharm. Inc., Princeton, NJ) and intermediate-duration insulin (Lente Purified Pork Insulin USP, Novo) was given subcutaneously, to control postoperative hyperglycemia. After stabilization of blood glucose levels, NPH long-duration insulin (NPH; Iletin II, Eli Lilly Co., Indianapolis, IN) was added to the regimen, as required to maintain fasting blood glucose (FBG) between 100 and 200 mg/dl. Pancreatic enzyme supplementation (Viokase-V, Fort Dodge Animal Health, Fort Dodge, IA) was added to the food at the dose of 10 g/day, to compensate for pancreatic exocrine insufficiency after Pct.

Experimental Design

Animals were randomly divided in five groups. After induction of diabetes by Pct, animals of group I ($n = 6$) underwent repeated islet allo-Tx into the liver, receiving the islets from two donors four times over 2 weeks. After Pct, animals of group II ($n = 3$) were transplanted with the same amount of islets, previously obtained from two donors, by a single intraportal injection. Animals of group III ($n = 3$) underwent Pct, followed by immediate intraportal islet auto-Tx. Animals of group IV ($n = 3$) underwent Pct and were used as diabetic controls. Group V animals ($n = 3$) were used as normal control pigs.

Transplantation of pancreatic islets was considered successful if FBG level was ≤150 mg/dl after withdrawal of insulin replacement therapy. Rejection was defined as FBG ≥250 mg/dl in three consecutive determinations. Animals were followed for glucose metabolism and body weight (BW) until euthanasia, which was performed 34 weeks after transplantation, or after ascertained rejection. At necropsy, the liver was resected and multiple tissue specimens were taken and processed for light microscopy, after standard hematoxylin and eosin staining.

Total Pancreatectomy

Anesthesia was induced in overnight fasted animals using ketamine (20 mg/kg, IM), acepromazine (0.6 mg/kg, IM), and atropine (0.05 mg/kg), and was maintained with thiopental (10 mg/kg, IV) and isoflurane/O_2 after oral intubation. Surgery was performed under sterile conditions. Antibiotic therapy with a broad-spectrum cephalosporine (Cefadyl, Bristol Lab. Div., Syracuse, NY, 2 g/day, IV) was carried out for 5 days, starting the day of surgery. Buprenorphine hydrochloride, 0.05 mg/kg IM (Buprenex, Reckitt & Colman Pharmaceuticals, Richmond, VA), was administered for pain the day of surgery and on postoperative day 1. For Pct, a conventional surgical technique was used (30). The abdomen was entered through a vertical midline incision. The peritoneum above the body and tail of the pancreas was opened, and the large venous branch from the splenic vein was identified and ligated. The tail of the pancreas was mobilized, starting close to the splenic hilus and heading toward the midline. The pancreatic artery and vein were ligated distally at the upper border of the gland. The pancreas was dissected free from the left adrenal gland and its vein, and separated from the portal vein by tying the connecting branches. The inferior pancreatic artery was ligated, and the pancreatic ring around the portal vein and the head of the pancreas was dis-

sected free from the right part of the portal vein and the infrahepatic vena cava. The pancreas was then separated from the duodenum, sparing the pancreatoduodenal artery by individual ligation of the pancreatic branches. The pancreatic duct was tied distally and the gland was removed. In animals undergoing islet Tx, the gastroduodenal vein was cannulated by an indwelling catheter connected to a subcutaneous reservoir (Port-A-Cath, Pharmacia Deltec Inc., St Paul, MN). Its chamber was filled with heparinized saline and placed in a subcutaneous pocket in the flank. A special jacket (Lomir, K.L.A.S.S., San Jose, CA) was used to avoid scratching injuries to the skin overlying the subcutaneous port.

Islet Preparation

Porcine islets were isolated by a modification of the standard collagenase digestion method (25). The pancreas was preserved in ice-cold University of Wisconsin solution for 1–2 h after harvesting. The gland was distended by intraductal injection of 700 mg collagenase (Type P, Boehringer Mannheim Co., Indianapolis, IN), dissolved in 350 ml of 2% newborn calf serum HEPES-buffered Hank's balanced salt solution (NCS-HEPES-HBSS, Sigma Chemical Co., St. Louis, MO), at 4°C. The pancreas was then loaded into a flask containing several marble bills and digested by incubation at 37°C, manually shaking every 3 min. The process was stopped by cold NCS-HEPES-HBSS addition after detecting free islets by dithizone staining, under an inverted microscope (20). The pancreatic digest was then washed and filtered through a 400 μm stainless steel wire mesh, in a 4-L, orbitally shaking, custom-made stainless steel chamber. For islet purification, the pancreatic digest was bottom loaded and centrifuged on a discontinuous Euro-Ficoll gradient, obtained by dissolving 500 g of Ficoll DL-400 (Sigma Chemical Co.) in 1.5 L of Euro-Collins perfusion solution (Fresenius AG., Bad Homburg v.d.H., Germany), at the final densities of 1.121, 1.091, and 1.063 g/ml, by using a COBE 2991 cell washer (Cobe, Lakewood, CO), as previously reported (19). The final islet preparation was suspended in CMRL-1066 (Sigma Chemical Co.), added with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, at 37°C. Evaluation of islet cell number, volume, and purity was estimated under an inverted microscope on dithizone-stained cells, and the islet equivalent (IE) number was calculated (26). Validation of the islet isolation method was obtained by reversal of diabetes through 2000 IE Tx under the kidney capsule of nude streptozotocin-diabetic mice (data not reported).

Islets were either transplanted immediately after isolation or cultured for 48 h, 10,000 IE in 50 ml 10% FCS-CMRL-1066/150 × 15 mm uncoated petri dish, at 37°C, in a 5% CO_2/95% air humidified atmosphere. Before Tx, residual islets were eventually quantified in the petri dishes, to verify complete islet retrieval.

Identification of Donor–Recipient Pairs

Alloreactive donor–recipient pairs were chosen based on positive mixed leucocyte culture (MLC) reactivity, with stimulation indices >6. Porcine peripheral blood lymphocytes (PBL) were isolated from heparinized peripheral blood via density gradient centrifugation over Ficoll-paque (Pharmacia, Piscataway, NJ). In a one-way MLC, 1×10^5 recipient PBL were used as responders against 1×10^5 γ-irradiated (3000 rad) donor PBL. Cultures were established in 96-well U-bottom tissue culture clusters. The medium consisted of RPMI-1640 (Sigma Chemical Co.), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L L-glutamine, 1× vitamins, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, and 10 mmol/L HEPES buffer (Sigma Chemical Co.). Cultures were incubated for 5 days in an atmosphere of 5% CO_2/95% air, at 37°C, pulsed with 2 μC of tritiated thymidine ([^{3}H]thymidine), incubated overnight, and harvested. Counts per minute (cpm) of incorporated [^{3}H]thymidine were determined, and data were expressed as cpm of recipient PBL versus irradiated donor PBL. The range of MLC stimulation indices for donor–recipient pairs used in this study was 6 to 45.5, reflecting a high degree of donor–recipient alloreactivity.

Islet Transplantation

Islets were injected by puncturing the subcutaneous reservoir with a 21-gauge, noncoring needle (Vastack, Gish Biomedical Inc., Irvine, CA). Either freshly isolated or 48-h cultured islets were washed and resuspended in 150 ml HBSS. Over a period of 10 min, islets were infused intraportally through the subcutaneous reservoir by gravity drainage, followed by rinse with 100 ml HBSS. Portal pressure was checked in all animals before and after islet infusion by an H_2O manometer, and showed only a transient increase immediately after Tx, returning rapidly to the baseline.

Group I animals received four allo-Tx of ≥80% pure islets, averaging 71,665 ± 13,638 IE. Islets were obtained from two donor animals, and were injected twice a week, half freshly isolated and half 48-h cultured, over a period of 11 days and for a total of 23,647 ± 1617 IE/kg BW. Group II animals received a single allo-Tx of ≥80% pure islets, which were obtained from two donors, half freshly isolated and half 48-h cultured, for a total of 22,416 ± 1124 IE/kg BW. Group III animals received an auto-Tx of 60–75% pure islets, averaging 2980 ± 424 IE/kg BW, by intraportal injection through the subcutaneous reservoir, a few hours after Pct and immediately after islet isolation.

Immunosuppression

Cyclosporine A (CsA; Sandoz AG, Basel, Switzerland) was started 4 days before allo-Tx, at a dose of 15 mg/kg IM, and gradually reduced to 4 mg/kg IM or 30 mg/kg PO (Sandimmune Neoral, Sandoz AG, Basel, Switzerland). CsA blood level was determined twice a week, using a polyclonal RIA method (Sandoz AG); the drug level was maintained >1000 ng/ml for the first 30 days post-Tx, and ≥250 ng/ml thereafter. In group I long-term normoglycemic animals, CsA treatment was discontinued after 12–13 weeks ($n = 2$) or after 22 weeks ($n = 1$).

Intravenous Glucose Tolerance Test (IVGTT)

IVGTT was performed 10 days after Pct, 30 days after islet transplantation, and then at monthly intervals. After overnight fasting, 0.5 g D-glucose/kg BW was administered via a peripheral vein. Plasma insulin and glucose levels were evaluated on blood collected at −10 and 0 min, and at 5, 10, 15, 20, 30, and 60 min postinjection. For statistical comparisons, the area under the curve (AUC) was calculated for each group of animals. For insulin determination, a ^{125}I-labeled porcine insulin radioimmunoassay kit was used (Novo BioLabs, Novo Laboratories Inc., Wilton, CT). The *K* value, expressed as percent decline/minute in blood glucose, was calculated as the slope of the log of blood glucose versus time during the IVGTT.

Statistical Analysis

Results are expressed as means ± SD, and one-way analysis of variance (ANOVA) was used for statistical comparisons between groups, with a level of significance of 5%.

RESULTS

Diabetes was induced in all recipient and control animals by Pct, with no postoperative mortality. After surgery, there was a sharp rise of FBG, maintained between limits by a 3/day insulin injection regimen. Insulin requirement of glucose-stabilized pigs was about 1.2 IU/kg BW.

The results of islet isolation are summarized in Table 1. The isolation method gave a consistent islet yield; 48-h culture did not significantly affect islet morphology and number. Two donors of the Hanford breed were required to obtain an estimated adequate IE mass for one recipient Yucatan mini pig. In animals of group I, FBG was normalized within 2–3 weeks from the first islet injection, and insulin independence was reached 1 week after the last islet Tx (Figs. 1 and 2). Blood glucose and insulin plasma levels during IVGTT performed at 30 days in groups I, IV, and V are presented in Figure 3. While there was no statistical difference between the insulin AUC of group I versus group V, the blood glucose AUC was significantly different (group I vs. group V: $p < 0.05$; group I vs. group IV: $p < 0.01$; group IV vs. group V: $p < 0.01$). The *K* value at 30 days was 1.627 ± 0.98 in group I pigs, 0.542 ± 0.61 in group IV diabetic controls, and 2.689 ± 0.44 in group V normal control animals ($p < 0.05$ between group I and V; $p < 0.01$ between groups I and IV and between groups IV and V).

Two group I normoglycemic pigs died of acute pneumonia by *Actinomyces pyogenes* at 33 and 112 days, respectively, and one animal became progressively hyperglycemic at 100 days (Fig. 1). In these pigs MLC showed that host PBL did not respond to donor PBL, but responded well to a pooled PBL population. In the remaining three pigs, FBG was normal, with an insulin requirement not exceeding 0.1–0.2 IU/kg BW, and two of them were completely insulin free for 9–12 weeks (Fig. 2). Discontinuation of CsA after 3 months in two group I animals resulted in prompt FBG increase (Fig. 2), and at necropsy, at 102 and 87 days, respectively, lymphocyte infiltration and islet destruction were observed at the portal spaces. In one group I pig, CsA was stopped after 151 days, and normoglycemia persisted until euthanasia, after 34 weeks. At histologic examination, it was possible to observe islet cells at the portal spaces surrounded, but not infiltrated, by lymphocytes (Fig. 4A). In long-lasting normoglycemic animals, BW was maintained at pre-Tx value, while in the animal surviving 35 weeks there was a progressive increase to about 110% of the original BW at 6 months from Tx.

In group II pigs, normoglycemia was only transiently reached within a few days from islet Tx. In these pigs,

Table 1. Pig Islet Isolation Results

	Total No. Islets	No. Islets/g Pancreas	Total No. IE (150 μm)	Total No. IE/g Pancreas	Purity* (%)
Prepurification	403,824.8 ± 175,630.9	5,709.5 ± 1,968.3	183,005.0 ± 106,082.5	2,628.9 ± 1,391.1	—
Postpurification	241,969.2 ± 74,435.2	3,240.8 ± 993.4	117,469.1 ± 48,803.8	1,605.5 ± 694.5	82.7 ± 7.1

Pancreas weight (g) was 75.7 ± 14.8 ($N = 26$). IE (islet equivalent) represents the estimated number of islets assuming an average diameter of 150 μm/islet. Data are presented as mean ± SD.
*Dithizone stained vs. unstained cells.

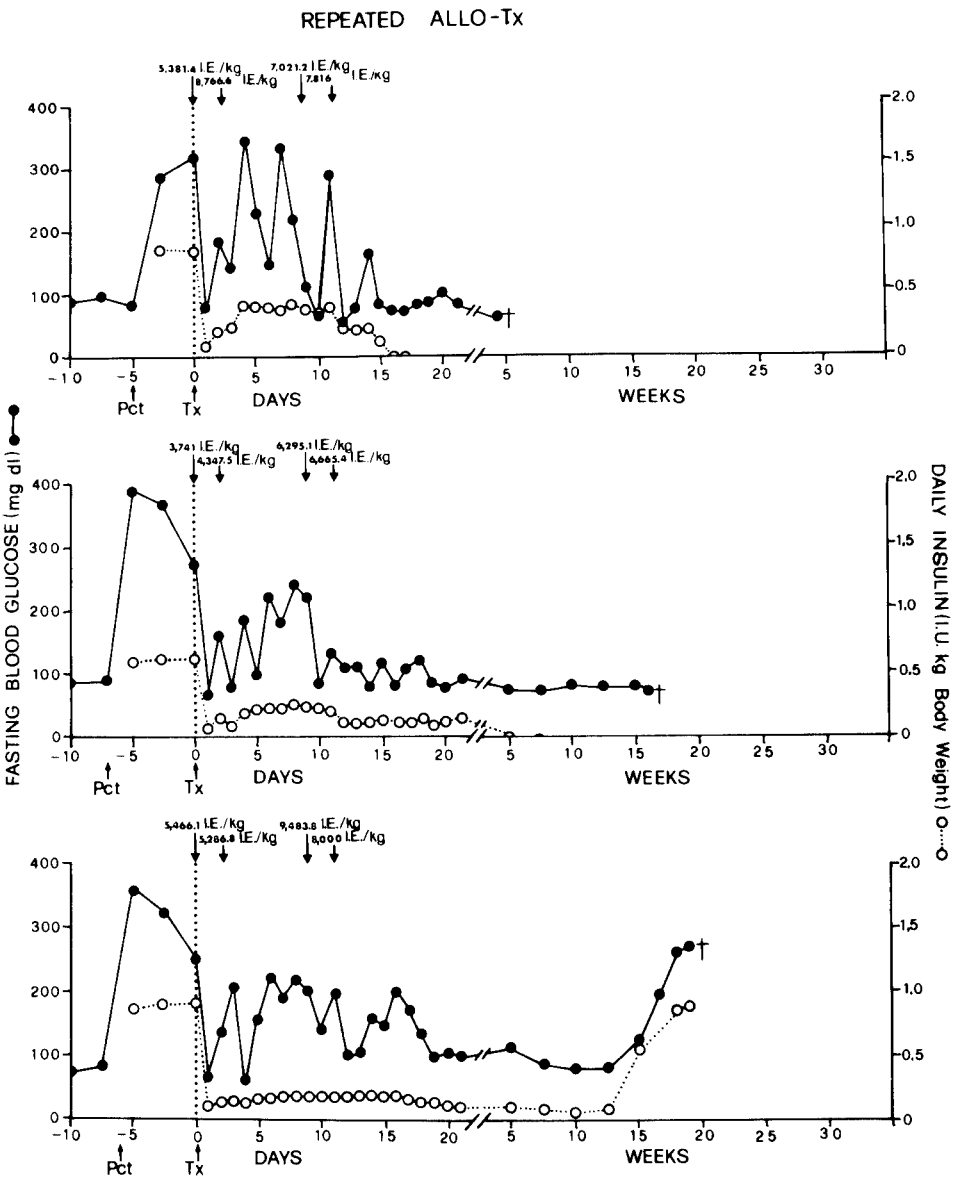

Figure 1. Time course of fasting blood glucose levels (●) and daily insulin requirements (○) in three totally pancreatectomized pigs submitted to repeated islet allo-Tx. Arrows indicate islet transplantation. The islet equivalents (IE)/kg body weight (BW) injected are reported for every infusion. In pig #1 (upper chart), 5381.4 IE/kg were injected (Tx) into the liver 5 days after total pancreatectomy (Pct), 8766.6 IE/kg were injected 48 h later, 7021.2 IE/kg after 9 days, and 7816 IE/kg after 11 days. Normoglycemia was reached on day 15 and insulin was withdrawn on day 17. This animal died on day 33 of acute pneumonia by *Actinomyces pyogenes* (†). In pig #2 (middle chart), 3741 IE/kg were injected (Tx) into the liver 7 days after Pct, 4347.5 IE/kg were injected 48 h later, 6295.1 IE/kg after 9 days, and 6665.4 IE/kg after 11 days. Normoglycemia was achieved on day 10, and insulin was withdrawn on day 35. The animal died on day 112 of acute pneumonia by *Actinomyces pyogenes* (†). In pig #3 (lower chart), 5466.1 IE/kg were injected (Tx) into the liver 6 days after Pct, 5286.8 IE/kg were injected 48 h later, 9438.8 IE/kg after 9 days, and 8000 IE/kg after 11 days. In this animal normoglycemia was reached on day 18, but insulin independence was not achieved; after 100 days, the pig became progressively hyperglycemic, and was killed after 18 weeks (†).

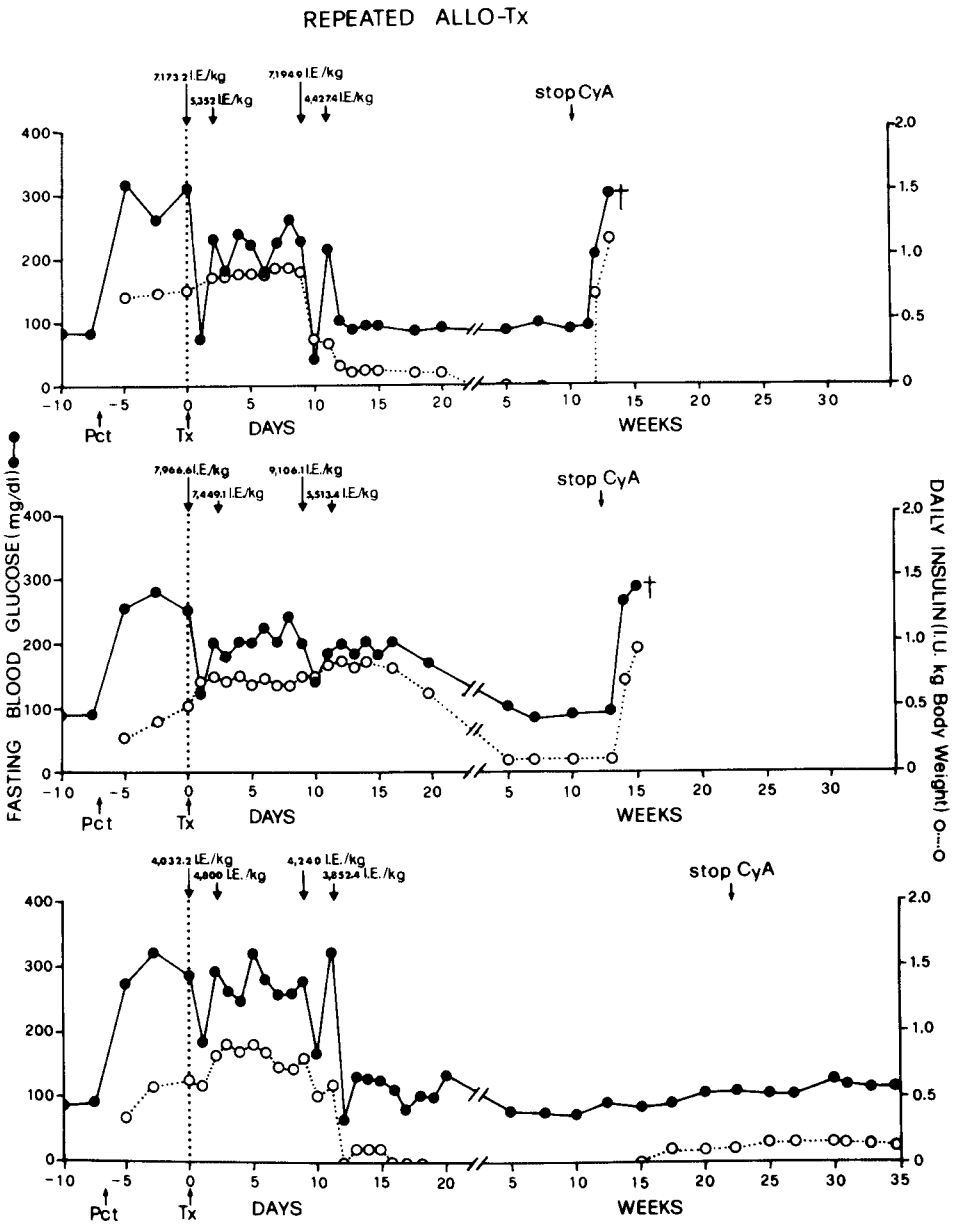

Figure 2. Time course of fasting blood glucose levels (●) and daily insulin requirements (○) in three totally pancreatectomized pigs submitted to repeated islet allo-Tx. In pig #4 (upper chart), 7173.2 IE/kg were injected (Tx) into the liver 7 days after Pct, 5352 IE/kg were injected 48 h later, 7194.9 IE/kg after 9 days, and 4427.4 IE/kg after 11 days. Normoglycemia was reached on day 12, and insulin was discontinued on day 35. After cyclosporine A (CyA) withdrawal, at 12 weeks, there was a prompt rise of fasting blood glucose levels, and the animal was killed after 13 weeks (†). In pig #5 (middle chart), 7966.6 IE/kg were injected (Tx) into the liver 7 days after Pct, 7449.1 IE/kg were injected 48 h later, 9106.1 IE/kg after 9 days, and 5513.4 IE/kg after 11 days. Normoglycemia was reached on day 35, but insulin independence was not completely achieved. After CyA withdrawal, at 13 weeks, there was a prompt rise of fasting blood glucose levels, and the animal was killed after 15 weeks (†). In pig #6 (lower chart), 4032.2 IE/kg were injected (Tx) into the liver 7 days after Pct, 4800 IE/kg were injected 48 h later, 4240 IE/kg after 9 days, and 3852.4 IE/kg after 11 days. In this animal normoglycemia was reached on day 12, and insulin was completely withdrawn on day 18. After 15 weeks, insulin was resumed at doses of 0.1–0.2 IU/kg BW. CyA was withdrawn at 22 weeks, and the pig remained normoglycemic until 34 weeks, when it was killed (†).

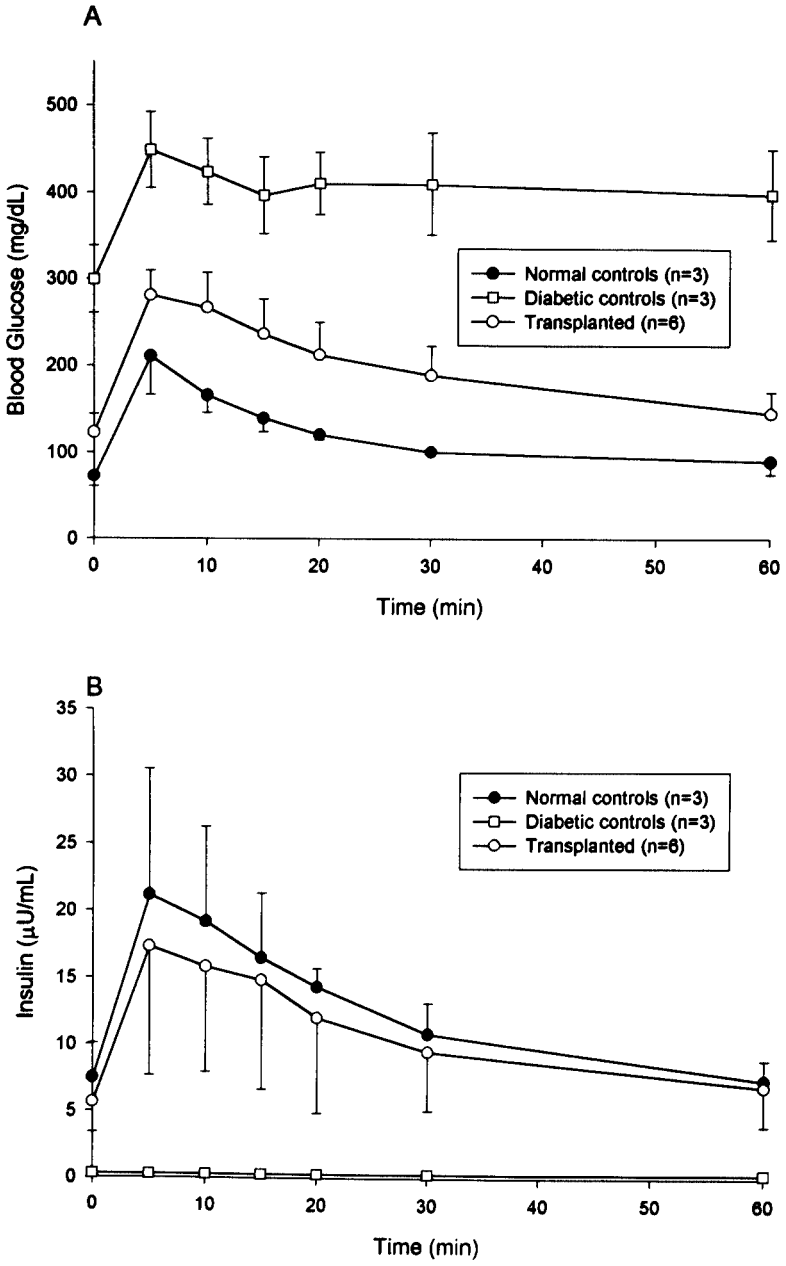

Figure 3. Results of IV glucose tolerance test (IVGTT) performed at 30 days in group I normoglycemic animals ($n = 6$), in group IV diabetic controls ($n = 3$), and in group V normal controls ($n = 3$). (A) Blood glucose profiles. The blood glucose area under the curve (AUC) was statistically different between groups (group I vs. group V: $p < 0.05$; group I vs. group IV: $p < 0.01$: group IV vs. group V: $p < 0.01$). (B) Insulin profiles. The difference between insulin AUC of group I and group V was not statistically different ($p > 0.05$). In group IV animals there was complete loss of insulin secretion.

FBG remained within limits for 4–20 days, with a progressive increase of insulin requirement thereafter (Fig. 5). At 40 ± 2 days from islet Tx, insulin requirement was not different from that of group IV diabetic control animals, and BW decreased of 10% by the end of the study period. At necropsy, no islet cells were found at the portal spaces, but only lymphocyte infiltration.

In group III pigs, normoglycemia lasted only 7–10 days. Daily insulin requirement progressively increased thereafter, and stabilized at 0.4 IU/kg per day, corre-

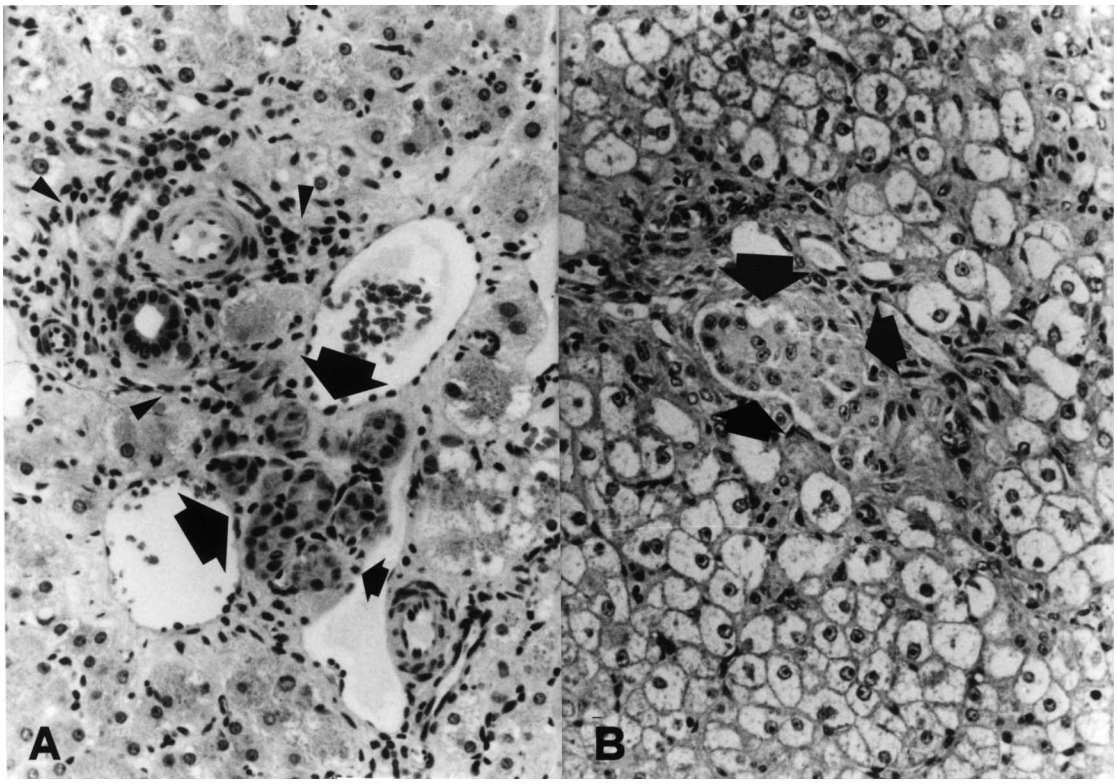

Figure 4. (A) Histological appearance of a porcine islet allograft (arrows), harvested 34 weeks after transplantation into the liver of a pig previously rendered diabetic by total pancreatectomy. The recipient animal was treated with cyclosporine A for 22 weeks after islet allotransplantation; thereafter, the immunosuppressive drug was suspended. Note the presence of few lymphocytes at the portal space site (arrowheads), surrounding but not invading the graft (H&E, original magnification ×200). (B) Histological appearance of a porcine islet graft (arrows) harvested 127 days after autotransplantation into the liver of a totally pancreatectomized pig. Hepatocytes surrounding the islet cells show prominent fatty vacuolization (H&E, original magnification ×200).

sponding to about one third of the amount of insulin required by group IV diabetic controls (Fig. 6). BW remained unchanged throughout the study period. At necropsy, morphologically intact islets were found at the portal spaces (Fig. 4B).

DISCUSSION

Clinical islet transplantation has been successful so far only in a minority of cases (1,6,12,32). A detailed analysis of the Islet Transplant Registry demonstrated that only 27% of 96 diabetic patients had a functional graft 1 year after islet Tx, and only 7% were insulin independent (14). However, restricting the analysis to the cases in which a sufficient islet mass (i.e., >6000 IE/kg recipient BW) had been transplanted intraportally within 8 h from pancreas harvest, and immunosuppression was achieved with the use of antilymphocyte/antithymocyte globulin, the percentage of success at 1 year increased to 70% for graft function, and to 20% for insulin independence, respectively (27). Further analysis of 24 islet grafts that fulfilled the above criteria revealed that 10 grafts failed in the early post-Tx period. Exclusion of these early graft losses from the computation resulted in a 1-year graft survival rate of about 80%, approaching this way the graft survival observed for solid organs (27). These considerations allow the conclusion that the functional islet mass engrafted at Tx plays a critical role for the long-term success of islet allo-Tx. Accordingly, methods aiming to improve immediate islet engraftment conditions are definitely needed, if early graft loss is to be avoided.

In the present study, islets were transplanted intraportally, because it has been shown that the liver represents the best site for islet Tx (15,17,22). Moreover, a tight glucose control in diabetic pigs was obtained through intensive insulin treatment, until post-Tx insulin independence, and this probably protected the islets from early exhaustion (4,13). When the islets were transplanted intraportally by a single injection, normoglycemia was obtained within 48 h, but lasted no more than 20 days. After 40 days from Tx, insulin requirement was not different from that of diabetic controls. In four out of six

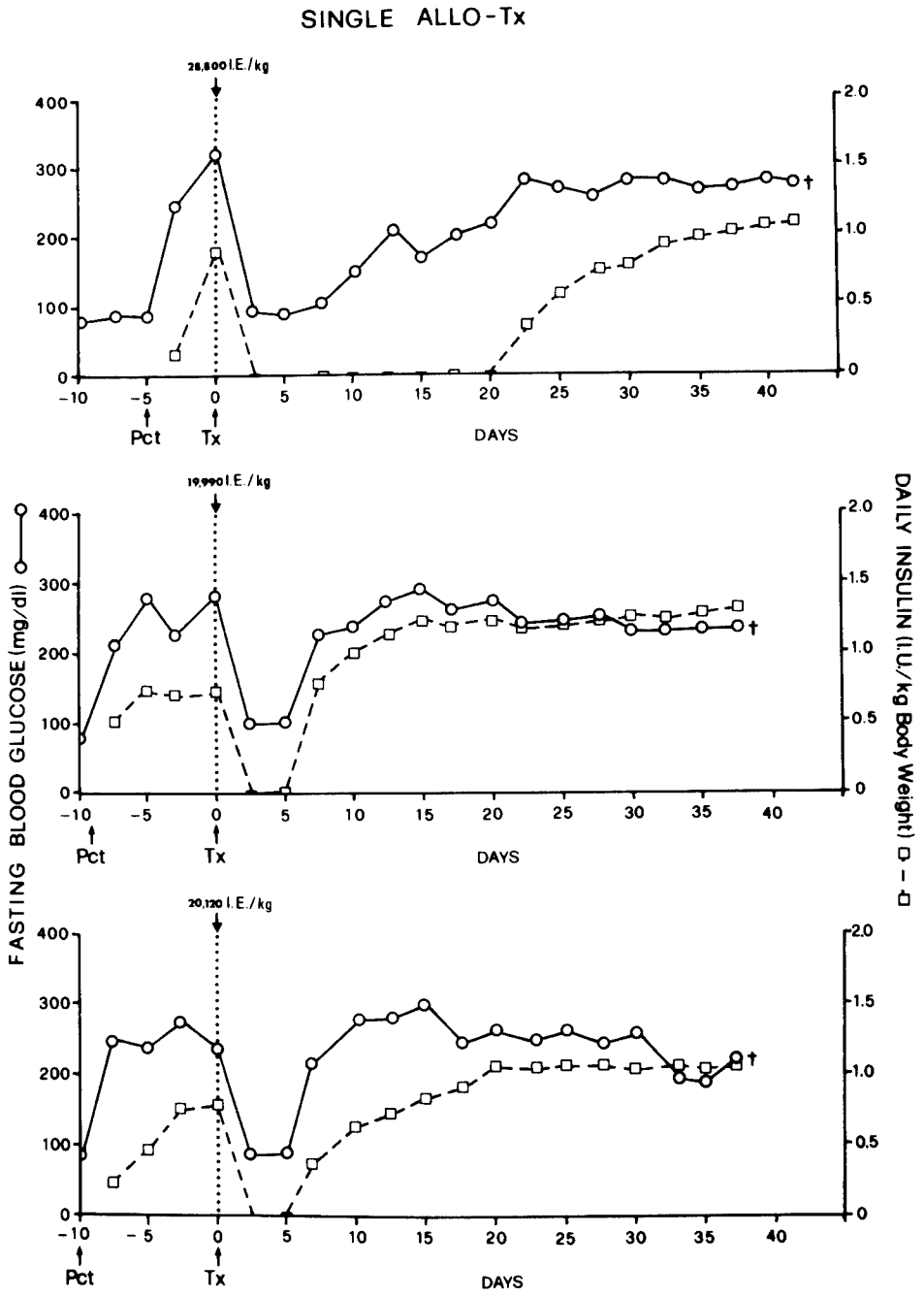

Figure 5. Time course of fasting blood glucose levels (○) and daily insulin requirements (□) in three totally pancreatectomized pigs submitted to single islet allo-Tx. In pig #1 (upper chart), 28,800 islet equivalents/kg (IE/kg) were injected (Tx) into the liver (arrow) at day 5 after total pancreatectomy (Pct). Normoglycemia lasted from day 3 to day 8, and then blood glucose level progressively increased, as well as insulin requirement. This animal was killed after 43 days (†). In pig #2 (middle chart), 19,900 IE/kg were injected (Tx) into the liver (arrow) 9 days after Pct. Normoglycemia lasted from day 3 to day 5, and then blood glucose level progressively increased, as well as insulin requirement. This animal was killed after 38 days (†). In pig #3 (lower chart), 20,120 IE/kg were injected (Tx) into the liver (arrows) 10 days after Pct. Normoglycemia lasted from day 3 to day 5, and then blood glucose level progressively increased, as well as insulin requirement. This animal was killed after 38 days (†).

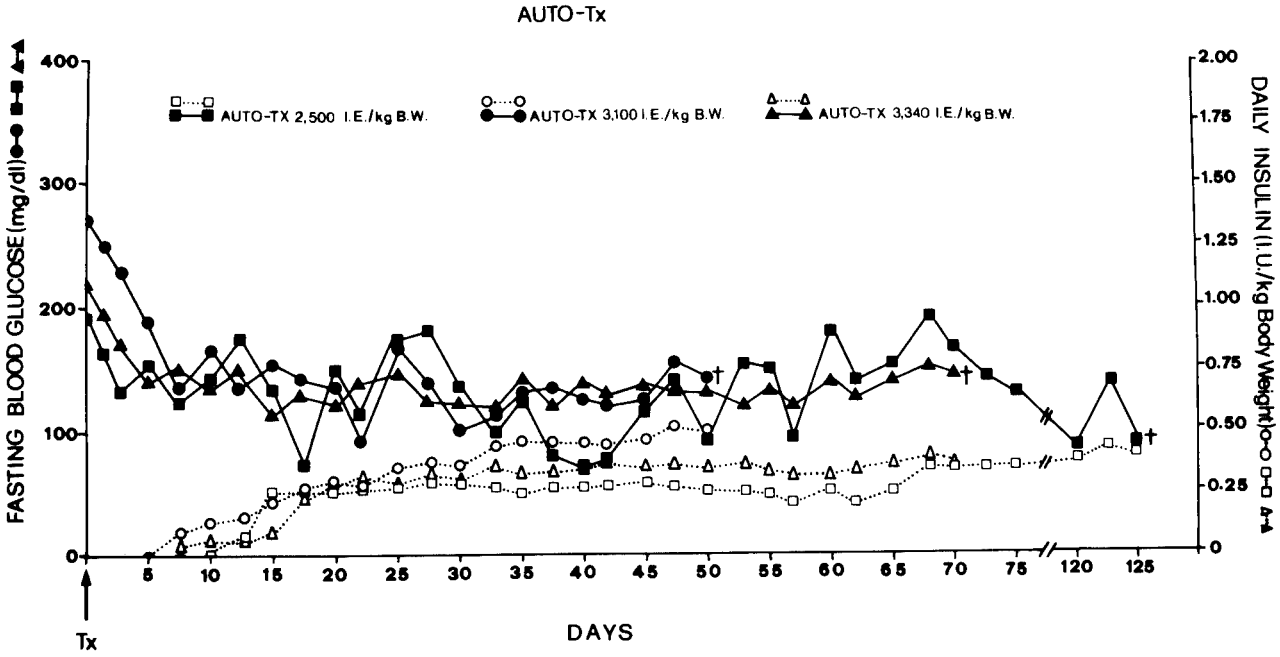

Figure 6. Time course of fasting blood glucose levels (solid lines) and daily insulin requirements (dotted lines) in three totally pancreatectomized pigs submitted to islet auto-Tx (arrow). Animals received 2500 islet equivalents/kg body weight (IE/kg BW), 3100 IE/kg BW, and 3340 IE/kg BW, respectively, a few hours after total pancreatectomy. Normoglycemia was reached after 7 days in all pigs, but insulin full independence was not achieved. Insulin requirement did not exceed 0.2–0.4 IU/kg. Animals were killed after 51 days, 72 days, and 127 days (†), respectively.

animals receiving multiple islet injections, insulin independence was reached within 1 week from the last Tx, and persisted until sacrifice or CsA withdrawal. Discontinuation of immunosuppressive therapy resulted in FBG increase in two out of three pigs, while one animal remained normoglycemic until sacrifice (i.e., 8 months after Tx and 3 months after withdrawal of CsA). In the auto-Tx group, insulin requirement was reduced to one third, and remained almost unchanged until euthanasia, but none of these animals became insulin free. Clearly, the islet mass obtained from a single pancreas did not reach the threshold required for insulin independence.

The single most important result in this series of experiments is that intraportal allo-Tx of a sufficient islet mass, infused in multiple subtherapeutic doses, produced a better metabolic long-term control in comparison to a single injection of the same amount of islets. This confirmed the results obtained in syngeneic rats by a similar experimental model (23). We hypothesized that a massive early loss of β cells following infusion of a large number of islets into the liver was a consequence of anoxia due to islet clumping at the portal spaces and thrombosis of small portal branches. Furthermore, some of the surviving islets could fail during the days following Tx, when revascularization is not yet completed (11). Eventually, a reduced number of well-engrafted islets may not be able to support the metabolic demand in a chronically hyperglycemic environment, leading to progressive β cell exhaustion (31).

An approach similar to our experimental protocol has been already described in dogs (8), as well as in humans (29). In a recently reported series of successful islet Txs, six out of seven patients received islets from two donor pancreases, and one recipient required a third Tx from two donors, under a glucocorticoid-free immunosuppressive regimen consisting of sirolimus, tacrolimus, and daclizumab (29). In our study too, no glucocorticoids were given. We used CsA at high dose, beginning 4 days before Tx, and maintaining a drug blood level >1000 ng/ml for the first 30 days post-Tx, because it has been shown that islet allograft survival could be consistently prolonged when immediate pretransplant CsA levels in serum exceeded 400 ng/ml (2). In our experience, likewise already reported in dogs (2), withdrawal of CsA therapy after 12 and 22 weeks resulted in persisting euglycemia in one out of three pigs, showing that a state of immune unresponsiveness to islet alloantigens had been induced, in spite of a high degree of initial donor/recipient alloreactivity. At this regard, there was no difference in donor/recipient alloreactivity between pigs undergoing islet rejection and the animal that remained euglycemic after CsA withdrawal. Pioneering work by Maki's

group showed that composite allografts made up of subtherapeutic numbers of rat islets prepared from multiple donors restored normoglycemia, when transplanted under the kidney capsule, suggesting a potential long-term immunological advantage of repeated islet Tx (10,16).

New therapeutic regimens aimed at inducing operational tolerance have recently been applied to nonhuman primates (5,18). The mechanism of porcine islet xenograft rejection is being further clarified by studies performed in the immunodeficient mouse model (9). If antirejection future strategies will prove successful, miniature swine inbred for the major histocompatibility complex (MHC) would be excellent candidates for islet donors, in that they are the only large animals from which one can harvest tissues for transplantation with defined MHC haplotypes (28). Until tolerance induction to xenotransplantation proves feasible, lack of islet tissue for transplantation will be the most important limitation to the wide applicability of this technique for the treatment of type I diabetes (33).

In conclusion, we achieved long-term insulin independence in totally pancreatectomized diabetic pigs by repeated subtherapeutic islet injections obtained from two donors, under sole CsA immunosuppression. The technique of multiple-donor repeated islet Tx may prove useful to overcome the problem of primary nonfunction or early graft failure, currently limiting the success of clinical islet Tx in most cases.

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