Rapid Publication

Successful Reversal of Streptozotocin-Induced Diabetes With Stable Allogeneic Islet Function in a Preclinical Model of Type 1 Diabetes

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The recent focus on islet transplantation as primary therapy for type 1 diabetes has heightened interest in the reversal of type 1 diabetes in preclinical models using minimal immunosuppression. Here, we demonstrated in a preclinical rhesus model a consistent reversal of all measured glycemic patterns of streptozotocin-induced type 1 diabetes. The model used single-donor islet transplantation with induction of operational tolerance. The term "operational tolerance" is used to indicate durable survival of single-donor major histocompatibility complex (MHC)-mismatched islet allografts without maintenance immunosuppressive therapy and without rejection or loss of functional islet mass or insulin secretory reserve. In this operational tolerance model, all immunosuppression was discontinued after day 14 posttransplant, and recipients recovered with excellent health. The operational tolerance induction protocol combined peritransplant anti-CD3 immunotoxin to deplete T-cells and 15-deoxyspergualin to arrest proinflammatory cytokine production and maturation of dendritic cells. T-cell deficiency was specific but temporary, in that T-cell–dependent responses in long-term survivors recovered to normal, and there was no evidence of increased susceptibility to infection. Anti-donor mixed lymphocyte reaction responses were positive in the long-term survivors, but all showed clear evidence of systemic T-helper 2 deviation, suggesting that an immunoregulatory rather than a deletional process underlies this operational tolerance model. This study provides the first evidence that operational tolerance can protect MHC nonhuman primate islets from rejection as well as loss of functional islet mass. Such an approach has potential to optimize individual recipient recovery from diabetes as well as permitting more widespread islet transplantation with the limited supply of donor islets. *Diabetes* 50: 1227–1236, 2001

Isolated pancreas islet allotransplantation (IPIT) has attracted recent attention as an imminently promising approach to achieve euglycemia and long-term relief from exogenous insulin therapy in patients with type 1 diabetes. Shapiro et al. (1) established proof of principle in a systematic series of seven type 1 diabetic patients at the University of Edmonton, Edmonton, Alberta. This group has now been expanded to 13 patients, the large majority of whom are insulin-independent at 1 year (Rajotte R, personal communication). Notably, prolonged reversal of type 1 diabetes with novel immunosuppressive and tolerogenic strategies has also been reported after isolated islet transplantation in four nonhuman primate series (2–5). Kenyon et al. (3,4) reported long-term survival in seven of seven baboon and six of six rhesus monkey recipients using monthly maintenance immunosuppressive therapy with anti-CD154 (hum58Biogen). Another study from our group reported rejection-free long-term survivors at 3 months to ≥1 year without any maintenance immunosuppressive therapy after concordant islet xenotransplantation and brief anti-CD3 immunotoxin (IT) and cyclosporine treatment in three of three spontaneously diabetic nonhuman primates (5,6). Most importantly, these primates showed durable and unchanged functional islet mass and normal acute insulin release (6).

In concert, these advances generated momentum for islet allotransplantation as a primary therapy for human type 1 diabetes. However, there are drawbacks to consider. There is an uncertain risk that allogeneic islet transplant recipients will undergo recurrent autoimmune disease (7). Additionally, there are complications of maintenance immunosuppressive therapy to control rejection, and there

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BG, blood glucose; DSG, 15-deoxyspergualin; ELISA, enzyme-linked immunoassay; FBS, fetal bovine serum; HBV, hepatitis B virus; IEQ, islet equivalent; IEQ_{150}, IEQ normalized to 150-μmol/l diameter; IFNγ, γ-interferon; IL, interleukin; IPIT, isolated pancreas islet transplant; IT, immunotoxin; IVGTT, intravenous glucose tolerance test; K_g, glucose disposal rate; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; NF, nuclear factor; R-D, recipient-donor; PBL, peripheral blood lymphocyte; PCR, polymerase chain reaction; PHA, phytohemagglutinin; RR, relative response; SI, stimulation index; SSP, sequence-specific primer; STZ, streptozotocin; TH, T-helper.

are requirements for retransplantation to offset loss of functional islet mass and insulin secretory reserve that occurs in both early and late follow-up after IPIT (1,8–11). To date, there have been no published reports of long-term function of single-donor major histocompatibility complex (MHC)-incompatible allogeneic IPIT for ≥1 year without maintenance immunosuppressive therapy and/or exogenous insulin in human IPIT recipients, and there has been only one report of operational tolerance in naturally diabetic nonhuman primate IPIT recipients (5). The healthy rejection-free course and stability of functional islet mass in the latter study (6) provided a rationale to examine tolerance induction to allogeneic IPIT in an inducible streptozotocin (STZ) preclinical type 1 diabetes nonhuman primate model.

We recently reported in nonhuman primate recipients of kidney allograft a novel approach to durable chronic rejection-free tolerance that persists for years without maintenance immunosuppressive therapy (12). This strategy used a short peritransplant treatment combination with anti-CD3 diphtheria-based IT. The IT depletes the lymphoid system of circulating and sessile T-cells (13) of both naïve and memory phenotypes (13a), whereas 15-deoxyspergualin (DSG) concomitantly blocks proinflammatory cytokine production and the maturation of dendritic cells by inhibiting nuclear translocation of nuclear factor (NF)-κB (14,15). The achievement of durable specific immune tolerance to kidney allografts with documented MHC class I and class II incompatible alleles (12) led us to postulate that prevention of islet allograft rejection and maintenance of stable functional islet mass on a long-term basis might also be achievable without maintenance immunosuppressive therapy using the IT plus DSG induction strategy.

This study examined a series of STZ-treated insulin-dependent diabetic rhesus macaques for the duration of insulin-free survival as well as the functional islet mass after single-donor MHC-incompatible IPIT. The results show stable long-term islet graft function in IPIT recipients without use of any exogenous insulin or maintenance immunosuppressive therapy after a 2-week tolerance induction protocol. Additionally, immunological studies provide evidence for immune competence in long-term survivors. Peripheral T-cells recovered fully after depletion by IT; numerous immune function studies affirm immune competence in long-term survivors and show a profound sustained T-helper (TH)-2–type cytokine deviation. These studies offer the first evidence in a preclinical nonhuman primate model that operational tolerance to a single-donor alloislet graft with multiple MHC incompatibilities in the absence of maintenance immunosuppressive therapy or exogenous insulin is achievable in rhesus monkeys with STZ-induced type 1 diabetes. The results provide a rationale for translational studies of operational tolerance induction for treatment of human type 1 diabetes.

RESEARCH DESIGN AND METHODS

Rhesus macaques. Recipients were pathogen-free juvenile (2–3 years old, 3.1–3.8 kg) male rhesus macaques obtained from Covance Research Products (Alice, TX). Pancreatic islet donors, obtained from Covance and LABS (Yemassee, SC), were 3–4 years old and weighed 3.5–5.5 kg. All animals had continuous water supply and were fed Harlan Primate Diet supplemented with fresh fruits twice daily. Monkey care and handling and the experiments described were performed in accordance with the Guide for Care and Use of Laboratory Animals (16) and were approved by the institutional animal care and use committee.

Restraint for bleeding and intravenous glucose tolerance test (IVGTT) procedures was achieved with an intramuscular injection of 10 mg/kg ketamine (Fort Dodge Laboratories, Fort Dodge, IA) mixed with 1 mg/kg acepromazine (Vedco Laboratories, St. Joseph, MO). Antibiotic treatment was cephazolin (Eli Lilly, Indianapolis, IN) given at 12.5 mg/kg i.m. twice daily on days 0 to +6 and oral vibramycin (Pfizer, Evanston, IL) given at 1.5 mg/kg on days +7 to +21. Oral Ensure Plus at 15 $mg \cdot kg^{-1} \cdot day^{-1}$ provided supplemental nutritional support for 2 weeks posttransplant. Buprenorphine hydrochloride (0.05 mg/kg i.m. every 12 h) (Reckit Colman Pharmaceuticals, Richmond, VA) was used for analgesia after surgery.

Immunosuppressive treatment. IPIT recipients were induced on the day of transplantation with one of three protocols, two of which used $F(Ab)_2$-IT, a conjugate of IgG or $F(Ab)_2$ fraction of FN18 anti-rhesus CD3ε mAb and CRM9 mutant diphtheria toxin. The three protocols included $F(Ab)_2$-IT alone, $F(Ab)_2$-IT plus DSG, or DSG alone. $F(Ab)_2$-IT was prepared by D.M.N., as described (14). In addition, all IPIT recipients had intravenous methylprednisolone (Upjohn, Kalmazoo, MI) administered at 7 $mg \cdot kg^{-1} \cdot day^{-1}$ given 4 h pretransplant and at 3.5 $mg \cdot kg^{-1} \cdot day^{-1}$ and 0.35 $mg \cdot kg^{-1} \cdot day^{-1}$ on the next 2 days, respectively. The first of two $F(Ab)_2$-IT infusions were administered intravenously as a 100 μg/kg bolus at 2–3 h pretransplant. For the second treatment, $F(Ab)_2$-IT at the same dose was infused on day +1. DSG (NKT-01; Bristol Myers, Princeton, NJ, and Nippon Kayaku, Tokyo) was administered at 2.5 $mg \cdot kg^{-1} \cdot day^{-1}$ i.v. beginning 4 h pretransplant and continuing through day +14. The DSG was reconstituted in saline, kept at 4°C, and administered within 72 h after reconstitution. Intravenous fluids (Abbott Labs, Abbott Park, IL) were administered on days 0–5 to maintain hydration. Other than the islet infusion, the recipients did not receive any blood or other cell transfusions. No immunosuppressive agents were given after day 14 posttransplant. Two STZ-induced diabetic animals received $F(Ab)_2$-IT plus DSG without IPIT.

Induction of type 1 diabetes with STZ. To induce type 1 diabetes, normal recipients were treated 1–4 weeks before IPIT with an intravenous bolus of STZ at 140 mg/kg. The STZ (Sigma, St. Louis, MO) was mixed with 5 ml citrate buffer and infused over 1 min. As previously reported, a single large (>140 mg/kg) dose, but not smaller single or multiple STZ doses, reliably induces a type 1 diabetic state that is both similar to human type 1 diabetes and associated with similar secondary complications (17,18).

By day 3 post-STZ, all animals exhibited hyperglycemia, with fasting blood glucose (BG) >250 mg/dl. Human insulin 70/30 (Lilly, Indianapolis, IN) was administered twice daily at 2–4 $U \cdot kg^{-1} \cdot day^{-1}$ adjusted to maintain nonfasting BG between 250 and 400 mg/dl. Low levels of stimulated C-peptide in all STZ-treated animals confirmed insulin deficiency. In addition, STZ-treated recipients exhibited an impaired glucose disposal rate (K_g) after a 500-mg i.v. glucose bolus. K_g values were obtained with a glucometer (Accuchek; Roche, Indianapolis, IN). C-peptide values were measured by the University of Alabama at Birmingham (UAB) Outreach Laboratory using a chemiluminescent immunoassay (Immulite; DPC, Los Angeles, CA) and were uniformly found to be <0.5 ng/ml in the STZ-diabetic subjects. Insulin was measured with an enzyme-linked immunoassay (ELISA) kit (Abbott Diagnostics, North Chicago, IL). The reagents used for C-peptide and insulin assays contained antihuman antibodies that cross-react with macaques. Acute insulin response was calculated after a 0.5-g/kg i.v. glucose bolus, with the resting insulin value subtracted as previously described (6,19). Glycemic parameters were compared with values of normal juvenile male rhesus monkeys in our colony.

Recipient-donor combinations. All recipient-donor (R-D) combinations underwent prospective molecular typing for rhesus macaque–specific MHC class I and II alleles using sequence-specific primer (SSP)–polymerase chain reaction (PCR), as previously described (20,21). Class I typing was restricted to A-locus alleles. The R-D combinations were selected to have multiple-donor MHC mismatches. The R-D MHC mismatches for each combination are listed in Table 1.

Isolation of donor islets. Donor islets were prepared by the semiautomated Ricordi technique for nonhuman primates as described by Kenyon et al. (3), with minor modifications. Equipment and reagents were treated or prepared, respectively, to minimize endotoxin contamination. Pancreata were perfused in situ with cold University of Wisconsin solution.

After catheterization of the pancreatic duct, the organ was distended through the duct with room-temperature Liberase HI (0.47 mg/ml) (Roche, Indianapolis, IN) dissolved in Hanks' balanced salt solution containing 1U/ml DNAase I (Sigma). Digestion in Liberase HI solution was performed for 15–20 min, at which time multiple dithizone-positive islets appeared with minimal contaminating acinar cells and cellular debris. Islets were washed in RPMI-1640 with 10% fetal bovine serum (FBS), suspended in Eurocollins (Mediatech, Indianapolis, IN) containing 20% FBS, and isolated in a COBE blood processor (COBE Laboratories, Lakewood, CO) by centrifugation on a dis-

TABLE 1
Allogenic isolated pancreas islet transplant results in STZ-induced diabetic rhesus macaques

Recipient	Treatment*	Insulin-free survival (days)	Islet IEQ × 10^3/kg	Donor MHC mismatches (n) Class IA locus	Class II DRB loci B1	Class II DRB loci Non-B1
97D027	$F(Ab)_2$-IT+DSG	>400	23	4	3	5
97D208	$F(Ab)_2$-IT+DSG	>400	19	1	2	1
97D291	$F(Ab)_2$-IT+DSG	>400	25	2	2	3
97D207	$F(Ab)_2$-IT+DSG	>400	25	0	1	2
97D001	$F(Ab)_2$-IT+DSG	353†	25	3	3	1
97D004	$F(Ab)_2$-IT+DSG	187‡	25	3	3	2
97D164	$F(Ab)_2$-IT+DSG	70†	23	1	2	3
97D052	DSG	16†	25	2	1	1
96C080	DSG	15†	25	2	1	1
97D480	$F(Ab)_2$-IT	70†	21	1	1	1
97D2153	$F(Ab)_2$-IT	23†	21	1	3	5

*Peritransplant $F(Ab)_2$-IT days 0 and 1 plus DSG days 0–14. Methylprednisolone was given to all groups in a tapered dose over days 0–2, with no other immunosuppression. †Rejection; ‡surgical (gastrointestinal) complication after cannulation of hepatic vessels and intrahepatic glucose tolerance test (died euglycemic).

continuous Euroficoll gradient (Mediatech). Layered solutions were 100 ml at 1.037 s.g., 125 ml at 1.096 s.g., and 250 ml at 1.108 s.g., respectively. Isolated islets were counted by the method of Kenyon et al. (3), adjusted to ~500 islet equivalents (IEQs) normalized to 150-μmol/l diameter (IEQ_{150}) per milliliter, and cultured overnight in CRML-1066 (Mediatech) containing 10% FBS at 24–28°C and 5% CO_2. After counting and scoring viability with ethidium bromide/acridine orange, the islets were washed in RPMI-1640 with 25 μmol/l HEPES and transplanted.

Islet transplantation. At laparotomy, intraportal infusion of islets was performed by gravity flow through a no. 8 French pediatric feeding tube placed in the portal vein. For this series, a mean 23,363 ± 2,157 IEQ_{150} was infused per kilogram over 15 min. None of the IPIT recipients were given exogenous insulin treatment

Monitoring IPIT function. Nonfasting BG was monitored three times weekly from morning tail-stick samples. Stimulated C-peptide levels in serum were sampled 15 min after a 3-s i.v. infusion of 500 mg dextrose. Assessment of functional islet mass was performed by IVGTT at serial intervals posttransplant. After an overnight fast, IVGTT was performed under low-dose ketamine for sedation. After baseline BG determination, 0.5 g glucose/kg was infused as 50% glucose over 1–2 min. Samples were taken for BG determinations at 1, 3, 5, 10, 15, 30, 60, 90, and 120 min and tested with a glucometer. K_g was calculated from IVGTT using the 10- and 30-min time points and is expressed as the percentage of glucose per minute (19,22). Acute insulin response to glucose was calculated from the sum of the 2-, 4-, and 5-min insulin release values after a 0.5-g/kg glucose infusion minus the mean of the preinfusion insulin levels.

Measurement of transhepatic and intrinsic pancreas production of insulin. In two long-term IPIT recipients, a triple cannulation technique was used to compare transhepatic and intrinsic pancreas–stimulated insulin secretion. Cannulae were placed in the hepatic veins, portal vein, and a peripheral vein. After a 1-g/kg glucose bolus, insulin levels were measured at 1, 3, 5, and 10 min to assess insulin secretion from the transhepatic circulation versus the intrinsic pancreas.

T-cell and B-cell phenotype analysis. Changes in peripheral blood T- and B-cells were tested by flow cytometry (EPICS Elite; Beckman Coulter, Miami, FL) before and at periodic intervals after $F(Ab)_2$-IT treatment to document T-cell recovery. CD3 cells were measured by flow cytometry (EPICS Elite; Coulter) on forward and side scatter–gated lymphocytes using anti-rhesus CD3ε (FN18-labeled with spectral red, custom conjugated by Southern Biotechnology Associates, Birmingham, AL). B-cells were detected with FITC-labeled anti-CD20 (Pharmingen, San Diego, CA). Total T- and B-cell levels were calculated as described (13).

Antibody responses. In vivo immune competence was assayed by antibody responses to several antigens. One was the environmental antigen streptolysin O. Additionally, long-term survivors at 8 months to 1 year posttransplant underwent primary vaccination (single injection) to hepatitis B (Recombivax HB; Merck, West Point, PA) and to polyvalent pneumococcal polysaccharide (Pheumovax 23; Merck). Antibody levels were tested by ELISA in the UAB Outreach Laboratory. The monkeys' sera were uniformly negative for IgG to hepatitis B virus (HBV) and negative or weak positive for IgM to pneumococcal antigen before immunization. Lastly, IgG alloantibody to frozen donor peripheral blood lymphocytes (PBLs) was tested at 3-, 6-, and 12-month intervals by flow cytometry crossmatch, as previously described (12).

Mixed lymphocyte reaction. Peripheral blood mononuclear cells from long-term survivors were isolated on 1.078 s.g. ficoll and tested for T-cell responsiveness to phytohemagglutinin (PHA) (Abbott Diagnostics, Abbott Park, IL) and to irradiated allogeneic stimulator cells from donor and third-party sources in 96-well cultures, as previously described (23).

RESULTS

Pretransplant diabetic status of STZ-treated recipients. Before IPIT, all STZ-treated monkeys exhibited elevated nonfasting BG levels (mean 343.4 mg/dl ± 92.6 SD) compared with their normal pre-STZ baselines (71 ± 12.6, $P < 0.01$ by t test). The pretransplant diabetic BG and IVGTT values are included in Figs. 1 and 2, respectively. Consistent with the development of diabetes, serum C-peptide levels became uniformly negative after STZ and before IPIT (<0.6 ng/ml) compared with normal pre-STZ values (range 0.9–4.1 ng/ml; data not shown). IVGTT results confirmed type 1 diabetes, indicating abnormally reduced BG clearance rates (range 0.3–0.8% glucose per minute) at all time points compared with normal rhesus K_g values in our colony (6). Acute insulin response to glucose, reflecting the insulin secretory reserve, was also markedly abnormal (3.6 ± 1.7 μU/dl vs. normal mean values of 38 ± 6.4 μU/dl insulin). Before IPIT, all STZ recipients required 2–4 units 70/30 insulin twice daily to maintain BG at 250–400 mg/dl. To evaluate the stability of STZ-induced type 1 diabetes after tolerance induction without IPIT, two STZ-induced diabetic animals were maintained for >1 year as controls. We observed no reversal of type 1 diabetes in these nontransplanted controls. Thus, single high-dose STZ treatment was effective in unvaryingly establishing type 1 diabetes in our juvenile rhesus macaques, confirming earlier experience by others with high-dose (>140 mg/kg) STZ-induced type 1 diabetes in monkeys (17,18).

Recovery of isolated allogeneic nonhuman primate islets. Semiautomated isolation with Liberase HI yielded high recovery and purity of nonhuman primate islets obtained from donor pancreata (9–11 g) and allowed relatively consistent IEQs for transplantation. The range of islet dose was 19,000–25,000 IEQ (mean 23,727 ± 3,226) per kilogram of recipient weight (Table 1). After overnight culture, islet viability and purity ranged from 95 to 98% and from 90 to 99%, respectively. Endotoxin levels in the procurement solution bath, digest effluents, and culture media were minimal, averaging 0.45 ± 0.31 IE/ml. No adverse effects or increases in portal blood pressure were observed during intraportal infusion of the islets into diabetic recipients.

Stable reversal of type 1 diabetes after MHC-incompatible islet transplants without maintenance immunosuppressive therapy. Within 72 h posttransplant, nonfasting BG levels fell to normal in 100% (11 of 11) of recipients. None received exogenous insulin after transplantation. In group 1, treated with peritransplant $F(Ab)_2$-IT plus DSG, seven of seven demonstrated prolonged insulin-free graft survival without maintenance immunosuppres-

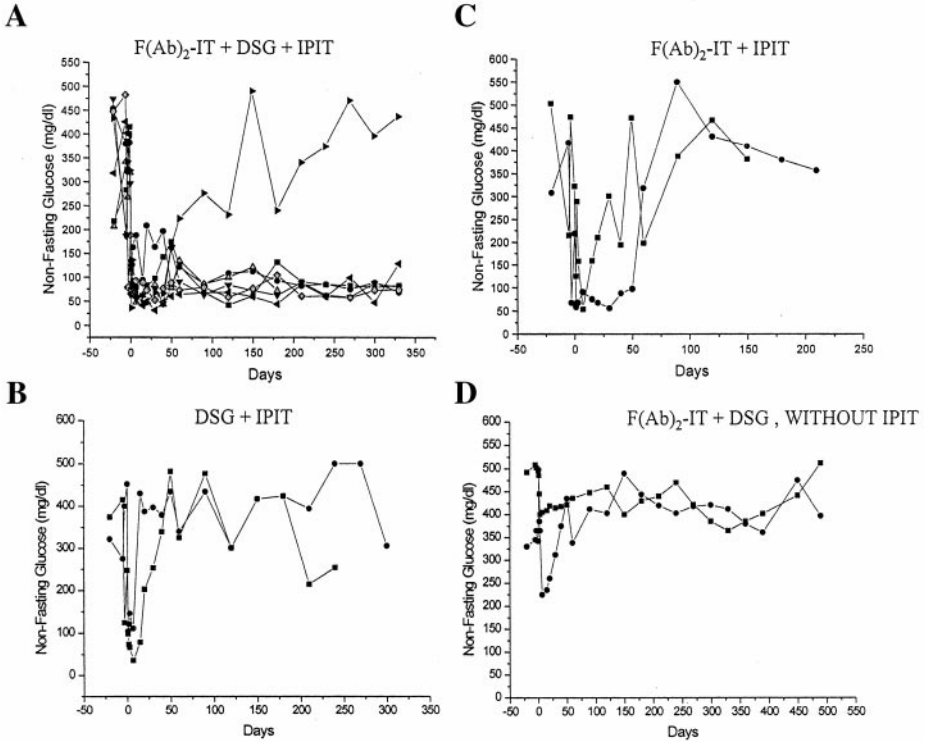

FIG. 1. Nonfasting BG levels before after IPIT. A total of 13 STZ-induced diabetic recipients were monitored for changes in BG. *A*: Of seven recipients given IPIT with IT plus DSG, six had stable BG without exogenous insulin during 1 year of follow-up. *B*: Two IPIT recipients given only DSG showed early IPIT function and subsequent rejection. *C*: Two IPIT recipients given $F(Ab)_2$-IT only had longer graft survival to 23 and 70 days, respectively. *D*: Two animals given $F(Ab)_2$-IT plus DSG without IPIT showed a stable diabetic state.

sive therapy (Table1). Four were euglycemic and insulin-free at >1-year posttransplant. At 353 days post-IPIT, the fifth recipient (97D001) was returned to low-dose insulin (50% or pre-IPIT dose) with loss of functional islet mass related either to late rejection or to other causes, possibly early islet apoptosis as previously described (24). Notably, this recipient had erratic early episodes of hyperglycemia. The sixth recipient (97D004) exhibited stable euglycemia for 187 days but died euglycemic from surgical complicaions following triple cannulation surgery to document in situ transplant islet function by transhepatic acute insulin release (shown in Fig. 3*A*). The seventh recipient rejected at 70 days. Thus, with the exception of the single rejection after 2-months post-IPIT survival, peritransplant induction with $F(Ab)_2$-IT plus DSG yielded an unprecedented result of long-term operational tolerance to unre-

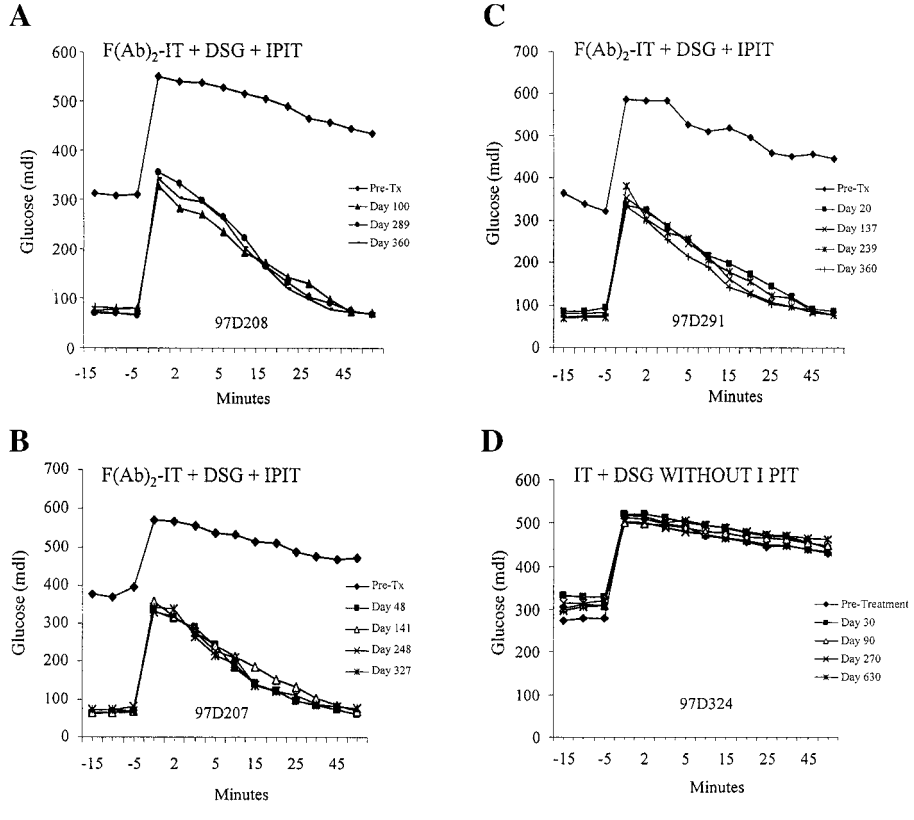

FIG. 2. IVGTT before and after IPIT. Serial IVGTT performed pretransplant and at later intervals ranging between 20 to 360 days posttransplant showed normal glucose decay curves returning to baseline within 25 min postglucose infusion, shown in long-term tolerant recipients. Individual IVGTT data on three of the long-term survivors are shown. 97D027 (*A*), 97D208 (*B*), and 97D291 (*C*). Pretransplant IVGTT data confirmed the diabetic state in all the STZ-treated recipients. *D*: IVGTT remained abnormal in the diabetic recipients given IT plus DSG induction without IPIT.

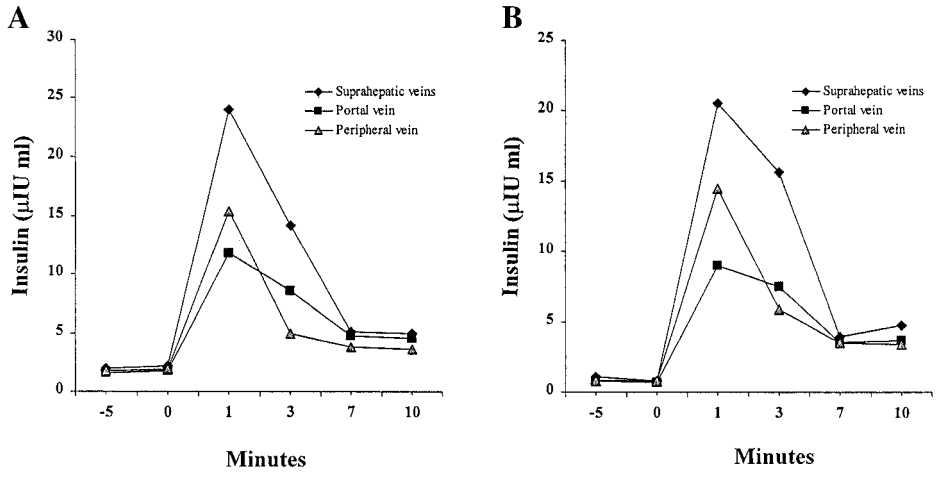

FIG. 3. Transhepatic acute insulin release. Glucose-stimulated transhepatic insulin release performed at 6 months post-IPIT in recipients 97D004 (*A*) and 97D001 (*B*). The data showed acute elevation in serum insulin levels in the suprahepatic veins compared with the portal vein and peripheral vein following peripheral intravenous infusion of glucose. These results demonstrated the function of the intrahepatic islet allografts.

lated single-donor allogeneic islets without maintenance immunosuppressive therapy or exogenous insulin.

In contrast to the group given $F(Ab)_2$-IT plus DSG, recipients in the two control groups, who were given $F(Ab)_2$-IT alone or DSG alone, failed to become long-term survivors. However, $F(Ab)_2$-IT treatment alone showed notable immunosuppressive activity, with one recipient surviving without insulin for 2 months. These results confirm that IT without an Fc fragment retains powerful immunosuppression but alone is not sufficient to induce stable operational tolerance (15). The multiple IPIT long-term survivors occurring after peritransplant treatment with $F(Ab)_2$-IT plus DSG without maintenance immunosuppressive therapy are consistent with a synergy between $F(Ab)_2$-IT and DSG for operational tolerance induction.

We used non-IPIT controls given IT plus DSG to exclude the possibility that IT plus DSG treatment might somehow reverse type 1 diabetes independent of the IPIT. Accordingly, we maintained two STZ-induced diabetic monkeys for >1 year after treatment with IT plus DSG alone without IPIT. Both animals remained insulin-dependent and diabetic without improvement in BG or functional islet mass (Figs. 1*D* and 2*D*). Therefore, IT plus DSG treatment alone clearly failed to reverse diabetes, confirming a requirement for IPIT to establish euglycemia.

In the current era, a claim of operational tolerance in nonhuman primates should be supported by evidence for MHC mismatching because histocompatibility can occur in outbred animals. Therefore, we used contemporary molecular MHC typing to prospectively insure MHC mismatches between the R-D combinations (20). All combinations had multiple MHC mismatches. In six of seven monkeys, both class I and II alleles were mismatched, and one of seven had several class II mismatches (Table 2). Therefore, chance genetic compatibility is not an explanation for the enduring insulin-free survival in these recipients.

Metabolic control in operationally tolerant IPIT recipients. To examine IPIT function, all recipients underwent serial BG tests and IVGTT. With the exception of occasional early episodic increases in BG in one recipient (97D001), BG values were in normal limits within hours after IPIT and remained so without rejection. Rejection was not treated, and, notably, only one acute rejection was observed at 70 days in one recipient (97D164) given $F(Ab)_2$-IT plus DSG. The DSG controls showed a rapid return to hyperglycemia at 2 weeks (Fig. 1*B*), whereas the $F(Ab)_2$-IT controls maintained normoglycemia for longer periods of 23 and 70 days, respectively (Fig. 1*C*). BG remained elevated in the non-IPIT recipients given IT plus DSG (Fig. 1*D*). Results in the group given $F(Ab)_2$-IT plus DSG were unique in that 85.7% (six of seven) developed stable BG levels for 1 year follow-up (Fig. 1*A*). In one animal, 97D004, follow-up was limited to 187 days, when he died from complications of the transhepatic acute insulin release procedure. Thus, stable IPIT function in the absence of maintenance immunosuppressive therapy was manifested by persisting normal BG levels in the long-term survivors.

To further evaluate IPIT function, IVGTT was performed at serial intervals in all groups to examine K_g. Six of seven long-term survivors in the operationally tolerant group exhibited stable normal K_g values after IPIT. Figures 2*A*–C represent individual longitudinal IVGTT profiles of three representatives of the five long-term survivors. The only exception in this group was 97D001, whose IVGTT became reproducibly abnormal at 353 days post-IPIT (data not shown) and resulted in his return to insulin therapy there-

TABLE 2
Allogeneic response in long-term IPIT recipients

Responder cells	Animals tested (*n*)	Mean response* to stimulators: Donor SI	Donor RR†	Unrelated third party SI	Unrelated third party RR†	PHA SI
LTS‡	6	34.6 ± 31.4	0.72 ± 0.31	43.7 ± 46.8	0.90 ± 0.40	399.0 ± 113.0
Normal controls	15	70.3 ± 51.4	0.77 ± 0.30	78.7 ± 20.1	0.76 ± 0.20	499.0 ± 96.0

Data are means ± SD. *No statistically significant difference was observed between the response of long-term survivors and controls; †long-term survivors were tested at ≥300 days post IPIT; ‡RR, relative ratio of the response to the specific donor or unrelated third party divided by the response to the unrelated pooled cell panel.

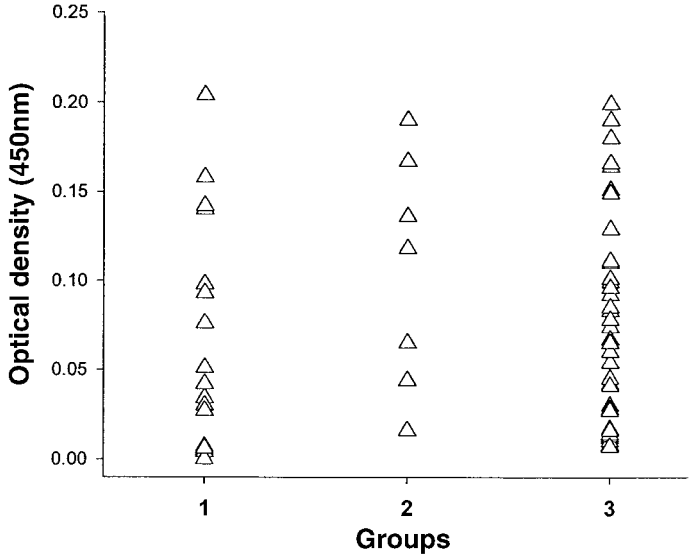

FIG. 4. Normal serum IgG antibody activity to environmental microbial antigen streptolysin-O. Results are presented as groups and show comparable antibody activity in the sera of monkeys tested by ELISA. The groups are normal untreated colony controls (group 1: $n = 15$, each tested × 1), age-matched STZ-induced diabetic animals (group 2: $n = 7$, each tested × 1), and 6-month posttreatment IPIT recipients given IT plus DSG (group 3: $n = 7$, each tested 4–5 times).

after, albeit at one-half the dose required pretransplant to maintain BG between 250 and 350 mg/dl. Figure 2*D* shows the IVGTT profile of a representative diabetic recipient given IT plus DSG without IPIT. Calculation of K_g values from IVGTT curves in the operationally tolerant group demonstrated values similar to those of normal rhesus monkeys in our laboratory, as previously reported (6). Likewise, acute insulin release was also unchanged in these long-term survivors (24a). Thus, the stable, normal IVGTT profiles >1 year are consistent with metabolic control and maintenance of a durable functional islet graft mass.

Transhepatic insulin release. Transplantation of the islets into the liver afforded the opportunity to directly assess their function in situ. Therefore, we tested transhepatic glucose–stimulated acute insulin release in two IPIT recipients (97D004 and 97D001) at 180 days, a time when both were euglycemic. The data in Figs. 3*A* and *B* show results of the transhepatic insulin release studies and indicate a twofold increase in insulin release in the suprahepatic veins compared with the portal vein following glucose infusion into a peripheral vein. These results provided evidence for functional islet mass within the liver and no demonstrable insulin release from the intrinsic pancreas.

IgG antibody to donor cells versus environmental microbial antigen. Despite the presence of multiple donor MHC mismatches and the absence of any maintenance immunosuppression, none of the recipients in any group exhibited either IgG- or IgM-positive flow cytometry anti-donor crossmatches during follow-up. The long-term survivor group was tested periodically at 2- to 3-month intervals through 1 year posttransplant (data not shown). To determine whether this quiescent humoral immune state in otherwise healthy animals reflected immune deficiency or immune regulation, we performed ELISA on multiple serum samples from the $F(Ab)_2$-IT plus DSG group to test for the presence of IgG to the environmental bacterial antigen streptolysin-O. Long-term survivors exhibited normal levels of IgG anti–streptolysin-O compared with normal rhesus monkeys and STZ-induced type 1 diabetes monkeys in our colony (Fig. 4). This result indicated that the long-term survivors, who were housed with the regular colony, were immunocompetent with respect to a common environmental microbial antigen (25). In this context, the absence of demonstrable anti-donor IgG to the abiding alloantigenic islets was seemingly specific. However, we did not attempt to validate the exact specificity of the allogeneic unresponsiveness, i.e., donor versus third party.

Immune response to vaccination. While the presence of anti–streptolysin-O antibody was consistent with a state of general immune competence, the stationary sampling times for these studies did not allow insights about possible immunoregulatory mechanisms that might be operational. Therefore, we conducted two different vaccinations in the long-term survivors at 300 days posttransplant and measured antibody responses as a function of time postimmunization. T-cell–independent IgM responses to pneumococcus vaccine were brisk and indistinguishable from the responses of normal controls over the 5–45-day follow-up period (Fig. 5). Thus, the kinetics and magnitude of the anti-pneumococcus response in long-term survivors were completely normal.

The T-cell dependent IgG antibody response to HBV, an antigen to which the monkeys had not been previously exposed, showed a different pattern. The ascending kinetic curve of IgG anti-HBV was indistinguishable from that of normal controls, indicating normal IgG response kinetics. However, the long-term survivors did not sustain the same level of antibody production as did the normal controls (Fig. 6). By 45 days after primary vaccination, the levels of IgG anti-HBV antibody were 6.8-fold reduced ($P < 0.05$) in the transplant recipients compared with their day 15 values and the day 15 and day 45 values of the normal controls. This suggested that there might be a negative regulatory process limiting T-cell amplification in the IPIT long-term survivors.

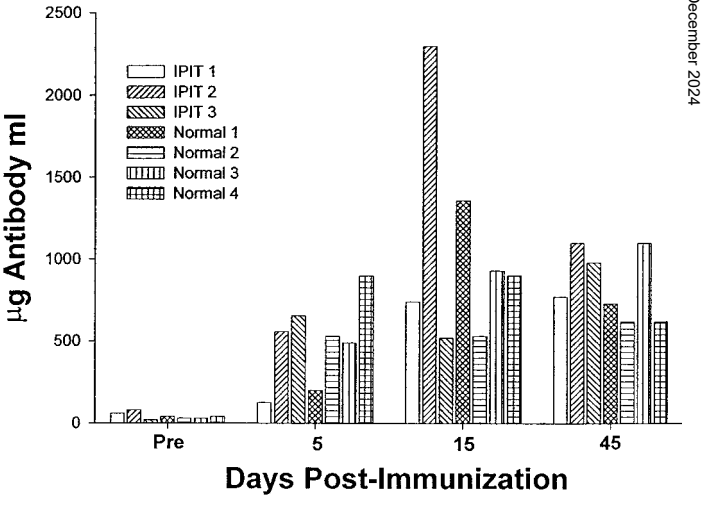

FIG. 5. Antibody response to pneumococcus vaccination. Serum antibody (IgM) response to primary vaccination with pneumovax was tested by ELISA before and at 5, 15, and 45 days postvaccination. The IPIT recipients numbered 1, 2, and 3 are all long-term survivors. The normal animals numbered 1–4 are nondiabetic colony controls.

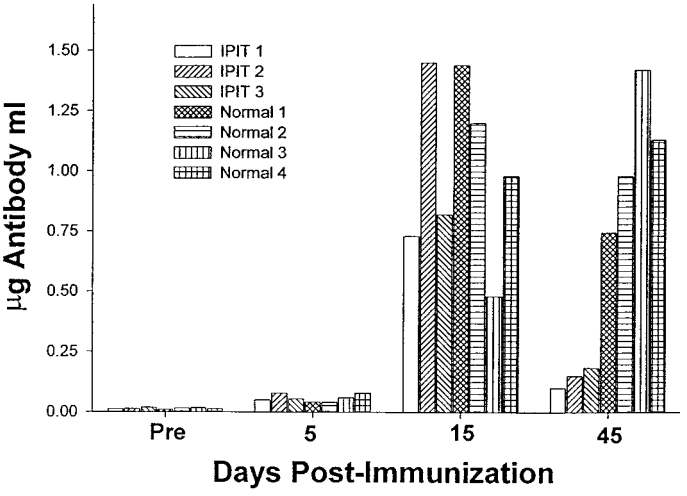

FIG. 6. IgG response to hepatitis B vaccination. Serum IgG antibody response to primary vaccination with hepatitis B was tested by ELISA at 5, 15, and 45 days postvaccination. The IPIT recipients and normal animals are the same as those represented in Fig. 5.

T-cell recovery and responsiveness to allogeneic cells or PHA is normal in long-term survivors. The peripheral T-cell population in long-term survivors was phenotypically normal. Similar to our findings reported for nonhuman primate kidney allograft recipients given IT plus DSG (13), percentages of peripheral T-cells in blood and lymph nodes of the seven IPIT recipients given $F(Ab)_2$IT plus DSG recovered from a nadir of <1% of pretransplant levels (54.3 ± 11.2%) to ~30% at 1 month and to full recovery (52.2 ± 11.4%) within 6–12 months posttransplant. Total T-cell counts followed a similar pattern.

To examine functional T-cell responsiveness in the 1-year IPIT survivors, we tested proliferating responses to PHA and to allogeneic cells in one-way mixed lymphocyte reaction (MLR). For MLR, we used cryopreserved stimulator cells from both the IPIT donor and an unrelated MHC-mismatched third party. Because we did not test pretransplant MLR responses to donor cryopreserved cells, we could not include a comparison of pre- and post-IPIT results. However, all were strongly positive to the donor in MLR. The T-cell responses of long-term survivors (n = 6) and normal controls (n = 15) were tabulated as group mean values ± standard deviation. The data are presented as the stimulation index (SI) obtained to the specific stimulator (i.e., donor or unrelated third party) and as the relative response (RR) to that stimulator divided by the response to a frozen pool of PBL that represents all DRB alleles present in the colony (Table 2). The mean anti-donor response (SI and RR) of the long-term IPIT recipients appeared lower than their response to unrelated third-party cells and the response of normal control cells to the donor, but these differences were not statistically significant by t test analysis (P = 0.684 and P = 0.156, respectively). Furthermore, although the long-term survivors' anti-donor, anti–third party, and anti-PHA SI values were ~40% lower than those of normal unrelated colony controls, these differences also did not reach statistical significance (P = 0.07). Thus, overall, the T-cell responses were consistent with intact donor reactivity as well as generalized immune competence. However, their discernibly reduced values suggested a possible systemic immunoregulatory process (e.g., TH-2–type cytokine deviation) might be restraining expansion of responding cells.

Systemic TH-2–type cytokine deviation in long-term survivors. A hallmark of IT plus DSG tolerance induction in our rhesus macaque kidney transplants is immune deviation associated with high levels of plasma interleukin (IL)-4 and low levels of γ-interferon (IFNγ) (12,14,15). The slightly reduced T-cell responses of the long-term survivors prompted us to measure their plasma cytokine levels. Compared with normal controls and STZ-induced diabetic animals, long-term survivors consistently exhibited enormously increased levels of IL-10 (mean 22-fold increase) and IL-4 (mean sixfold increase), whereas IFNγ was normal (Table 3). Like the kidney transplant recipients, analysis of early plasma samples in the IPIT recipients revealed heightened IL-4 and IL-10 levels and low IFN-γ levels within 1–2 weeks posttransplant (data not shown). Thus, induction with $F(Ab)_2$-IT plus DSG without maintenance immunosuppressive therapy was associated with prominent and sustained expression of systemic IL-4 and IL-10 for at least 12 months. This result is consistent with a TH-2 cytokine deviation response, which could be a factor in downregulation of peripheral T-cell responses measured in vitro and in vivo.

DISCUSSION

This study demonstrates for the first time, to our knowledge, the induction of stable insulin-free long-term operational tolerance in diabetic nonhuman primates given a single-donor allogeneic islet transplant with multiple MHC incompatibilities. Notably, all IPIT recipients given $F(Ab)_2$-IT plus DSG experienced prolonged graft survival without maintenance immunosuppressive therapy or exogenous insulin and several (four of seven) are currently >1-year survivors in excellent health with documented normoglycemia and immune competence.

Sharp et al. (26) first demonstrated the potential of the STZ-induction model for diabetes studies in primates. The studies of Theriault et al. (17) and Jonasson et al. (18) meticulously characterized glycemic parameters in STZ-induced cynomolgus and rhesus macaques, respectively. Jonasson et al. (18) also detailed secondary complications in an 11-year follow up of rhesus STZ-induced type 1 diabetes. With the caveat that evidence for autoimmune involvement is lacking, the STZ diabetic model in nonhuman primates has been well characterized and is arguably the best available preclinical model.

The ultimate goal of IPIT continues to be early treatment of juvenile diabetic patients in a safe and relatively noninvasive manner before chronic type 1 diabetes complications occur. The Edmonton study recently demonstrated that successful islet transplantation can reverse metabolic abnormalities of type 1 diabetes in a high per-

TABLE 3
Plasma cytokines show TH2 deviation in long-term IPIT recipients

NHP group	n	IL-4	IL-10	IFNγ
Normal	7	15.9 ± 4.4	40.5 ± 0.1	3.1 ± 0.7
STZ diabetic	4	13.2 ± 3.6	29.7 ± 4.3	2.1 ± 0.8
LTS IPIT 8–15	5	94.1 ± 44.9*	919.8 ± 326*	2.9 ± 0.6

Data are mean ± SD pg/ml of plasma. *LTS vs. normal: IL-4, P = 0.02; IL-10, P = 0.006 (Wilcoxon).

centage of humans for >1 year (1). These encouraging results have focused attention on preclinical nonhuman primate models that can provide proof of principle for defining optimal clinical translation. The findings of the Edmonton study point out two major barriers to optimal IPIT: limitations in current immunosuppressive protocols and the need to maintain functional islet mass and insulin secretory reserve, a factor that others have emphasized (27). Over a long period, the complications of maintenance immunosuppressive therapy mimic many of the multiorgan complications of type 1 diabetes (28). Thus, tolerance induction to avoid maintenance immunosuppressive therapy may be a pivotal factor in securing long-term success of IPIT.

There are few reported studies in which IPIT has reversed type 1 diabetes in nonhuman primates. Kenyon et al. (3,4) reported long-term survival after IPIT with the use of humanized anti-CD40L antibody (Hu5c8) in pancreatectomized rhesus monkeys and baboons. Using only monthly maintenance immunosuppressive therapy with anti-CD154 after the initial 2- to 3-week posttransplant induction period, these investigators showed functional allograft survival extending for >1 year in three recipients of primary IPIT. Rejection episodes were noted in all animals but could be successfully reversed in most recipients by additional Hu5c8 therapy. Rejection episodes, however, were associated with progressive loss of functional islet mass (4), a problem also associated with low rates of insulin-independent long-term human survivors (29). Thus, rejection episodes in IPIT appear to compromise long-term graft function. In this context, the absence of acute rejection in all long-term survivors from the IT plus DSG–treated group in the present study is probably a major factor in maintaining durable functional islet mass.

In 1999, we reported long-term survival up to 1 year in a group of spontaneously diabetic primates given xenogeneic IPIT and peritransplant IT with 4 days of cyclosporine (5). Those insulinopenic diabetic recipients showed an abnormal glycemic pattern with only trace levels of C-peptide (<0.5 ng/ml) (a requirement for exogenous insulin) and poorly controlled diabetes with ketosis, a profile comparable to the STZ-diabetic animals described in this report. Of note, the xenogeneic IPIT, like the allogeneic IPIT, functioned promptly without exogenous insulin, despite intrahepatic islet placement, which has been regarded by some as damaging to the islets (30). Thus, in our two studies of IPIT, induced each time with peritransplant IT, intrahepatic transplantation in nonhuman primates did not appear to compromise IPIT function.

The data in this report are clearly consistent with induction of operational tolerance to nonhuman primate islet allografts with defined MHC incompatibilities. Our earlier report of concordant nonhuman primate islet xenografts is arguably the first demonstration of operational tolerance in nonhuman primate islet transplantation (8). Importantly, no maintenance immunosuppressive therapy was given in either study, and yet acute rejection episodes were rare. The singular acute graft loss in the current study was the untreated allograft rejection at 70 days noted in the $F(Ab)_2$-IT plus DSG group. The reason for this rejection is uncertain, although it is worth noting that this recipient, unlike the others in the group, did not develop high levels of IL-10 and IL-4, a profile that might reflect a problem with DSG treatment or perhaps a difference in cytokine response genes.

An important finding was the stable functional islet mass present at all times posttransplant. The loss of functional islet mass after human IPIT is not well understood, but postulated autoimmune mechanisms might be a contributing factor (7). That STZ is not known to induce autoimmunity is a preclinical limitation of the STZ model. Although the etiology of nonhuman primate spontaneous diabetes in our earlier xenogeneic IPIT study using IT induction is uncertain, the metabolic abnormalities mimic those of spontaneous diabetic nonhuman primates and have been proposed to have an autoimmune basis (31). In this context, xenograft resistance to autoimmunity might have favored durable functional islet mass in xenogeneic IPIT, as suggested from studies in NOD recipients (32). This matter warrants further study of IPIT in spontaneously diabetic nonhuman primates using the current operational tolerance induction strategy and including both MHC-mismatched and -matched allogeneic IPIT. Of note, recent evidence showing cytoreduction in the memory T-cell population after IT plus DSG treatment (13a) bolsters the rationale for examining the ability of this strategy as a means to curtail recurrent autoimmunity as well as rejection within the allogeneic IPIT setting.

In the present report, we documented a full return of measured immunocompetence within months after operational tolerance induction. In addition to recovery of the T-cell mass, long-term survivors were tested for immune responsiveness to T- and B-cell–dependent antigens. The IgG antibody levels to the environmental bacterial antigen streptolysin-O and the normal kinetics of the response to pneumococcal vaccination are consistent with immune competence to bacterial antigens and may explain the clinical infection-free course of the long-term survivors.

The brisk IgG response to T-cell–dependent HBV vaccination and the results of in vitro MLR assays in long-term survivors at ≥300 days without maintenance immunosuppressive therapy confirm generalized T-cell competence. Furthermore, despite a state of stable ≥1-year reversal of diabetes without maintenance immunosuppressive therapy or exogenous insulin, the MLR results suggest that a state of donor-specific hyporesponsiveness did not develop in long-term survivors. Our finding of positive anti-donor MLR in recipients with durable IPIT acceptance and functional islet mass for ≥1-year without maintenance immunosuppressive therapy contrasts with observations of specific MLR hyporesponsiveness in other rhesus macaque IPIT studies using costimulatory blockade strategies that did not yield durable operational tolerance and stable functional islet mass (2,3). Although the basis for this difference is uncertain, our data suggest unresponsiveness to the direct pathway of donor antigen presentation is not sine qua non for durable operational tolerance to IPIT.

Deriving an evidence-based uniform immunological definition of transplant tolerance has been challenging in outbred nonhuman primate models. In addition to excluding clonal deletion of directly alloreactive cells, the anti-donor MLR responses observed in long-term survivors after recovery from a temporarily disordered lymph node architecture are consistent with tolerance mechanisms

involving immune ignorance (33) and/or peripheral suppressive mechanisms (34). MLR-induced activation is primarily dependent on recognition of MHC class II antigens, which are not normally expressed on human β-cells, acinar, or ductal cells in isolated islets (35). Furthermore, DSG treatment blocks maturation of class II+ dendritic cells that might have been transplanted with the islets (15). Thus, it is likely that the host immune system was not exposed to mismatched MHC class II antigens presented on donor islet T-cells, which could explain the retention of direct anti-donor MLR reactivity. This viewpoint is consistent with a recent report questioning the notion that anti-donor hyporesponsiveness in bulk MLC and/or cell-mediated lympholysis can be expected to correlate with tolerance in primarily nonvascularized grafts (36).

The high levels of the TH-2–type cytokines IL-4 and IL-10 without an increase in IFNγ, the prototype TH-1–type cytokine, suggest that a state of TH-2 cytokine deviation plays a role in operational tolerance in this IPIT model. The role of TH-2 deviation in tolerance induction is a provocative issue and has been actively debated (37,38), although studies in murine allogeneic IPIT suggest a beneficial effect of TH-2 deviation in prolonging islet engraftment (39,40). It is conceivable, furthermore, that chronic exposure to high systemic levels of IL-10, a cytokine that negatively regulates antigen presentation (41,42), might explain the slightly reduced MLR and PHA responses in our long-term survivors and their prompt suspension of anti-HBV production after the crest response to a single vaccination.

The matter of immune recovery following tolerance induction has been a major concern of our group, especially after the profound T-cell depletion in both circulating blood and sessile lymphoid populations after treatment with IT (13). The studies in allogeneic IPIT recipients demonstrate no sustained defects in long-term reconstitution of immune competence or the T-cell repertoire (13a). In the context of potential clinical application of operational tolerance to treat type 1 diabetes, this is an important issue because diabetic patients have a propensity to develop infections.

Overall, the long-term function of IPIT without maintenance immunosuppressive therapy, exogenous insulin, or evidence of compromised general immunity in STZ-induced diabetic nonhuman primates provides a stimulus for prudent expansion of human IPIT studies toward tolerance induction. These studies suggest that the goal of early islet transplantation for juvenile diabetes before development of secondary complications and without infections or maintenance immunosuppressive therapy may be close at hand with the use of operational tolerance protocols.

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