Indoleamine 2,3-Dioxygenase (IDO) catalyzes the breakdown of the amino acid tryptophan into kynurenine. It has been shown that IDO production by placental trophoblasts prevents the attack of maternal T-cells activated in response to the paternal HLA alleles expressed by the tissues of the fetus. In this article, we show that adenoviral gene transfer of IDO to pancreatic islets can sufficiently deplete culture media of tryptophan and consequently inhibit the proliferation of T-cells in vitro. Experiments in vivo have also demonstrated that transplantation of IDO-expressing islets from prediabetic NOD mouse donors into NOD-scid recipient mice is associated with a prolongation in islet graft survival after adoptive transfer of NOD diabetogenic T-cells. This protection is attributed to the depletion of tryptophan at the transplantation site beneath the kidney capsule. These results suggest that local modulation of tryptophan catabolism may be a means of facilitating islet transplantation as a therapy for type 1 diabetes. *Diabetes* 51:356–365, 2002

**C**linical expression of type 1 diabetes is the end point of a series of events occurring in genetically susceptible individuals (1). Type 1 diabetes can be considered a T-cell–mediated autoimmune disease that results in the destruction of the insulin-producing β-cells of the pancreatic islets of Langhans (2). The only treatment currently available for type 1 diabetic patients is that based on repeated daily injections of insulin. However, despite rigorous insulin replacement therapies, patients are unable to adequately regulate blood glucose levels to completely prevent the serious long-term consequences of hyperglycemia, including premature blindness, end-stage renal disease, and vascular and neuropathic disease (3). Because a complete understanding of the cause(s) of diabetes is still beyond our reach, a successful prevention strategy has yet to be found (4). Transplantation of the insulin-producing islet cells of the pancreas, requiring only a minor surgical procedure, is one way to replace the efficient insulin regulation that is lost in young type 1 diabetic patients (5). To date, successful pancreas or islet transplantation, avoiding both recurrence of autoimmunity and allorejection, has not been achieved without the help of an immunosuppressive regimen (6). Genetic modification of islets ex vivo by gene therapy approaches followed by transplantation is one promising means of efficiently replacing the physiological control of glucose metabolism (7).

Catabolism of tryptophan has been shown to play an important role in the immune response by inhibiting T-cell proliferation (8). Localized depletion of tryptophan in vivo has also been implicated in the immune evasion of certain tumors (9,10). Indoleamine 2,3-dioxygenase (IDO), the enzyme that regulates the rate-limiting step involved in the catabolism of tryptophan to kynurenine, is also involved in the control of microbial infections (11–14) and has been shown to have antioxidant properties (15–17). Pregnancy maintenance has also been associated with tryptophan catabolism by preventing the proliferation of maternal T-cells in response to tissues of the fetus expressing HLA alleles of the father (18). Localized expression of IDO seems then to result in tryptophan depletion and in a decrease in T-cell reactivity that is limited to the microenvironment surrounding the tissue of expression. The rest of the immune system remains intact. Consequently, the introduction of the IDO gene into pancreatic islets may provide an efficient immunological shield able to protect the β-cells from the diabetogenic autoimmune process and potentially promote the survival of allogeneic islets as a therapy for type 1 diabetes. In this study, we used an adenoviral vector encoding IDO to infect intact NOD mouse (19) islets to investigate its potential for local immunomodulation both in vitro and in vivo. These studies performed using the immunodeficient NODscid mouse (20) as transplant recipients demonstrated that expression of IDO in transplanted islets extended their survival time after adoptive transfer of diabetogenic T-cells.

**RESEARCH DESIGN AND METHODS**

**Animals.** All mice used were females purchased from The Jackson Laboratories (Bar Harbor, ME). BALB/c, C57BL/6, and NODscid mice (20) were used at 6–12 weeks of age. Prediabetic NOD (19) females aged 3–5 weeks were used as islet donors. NODscid transplant recipients were monitored three...
times a week for the presence of hyperglycemia. Diabetic NOD females, from the ages of 12–20 weeks, were used within 2 weeks of disease onset as a source of spleen cells for in vitro experiments and in vivo adoptive T-cell transfers. Mice were housed under specific pathogen-free conditions at the Children's Hospital of Pittsburgh Rangos Research Center Animal Facility, and all protocols were approved by the institutional Animal Research Care Committee.

Cloning of the murine IDO gene and RT-PCR detection. BALB/c mouse peritoneal macrophages (10 × 10^6) were cultured for 48 h in the presence of 500 units/ml γ-interferon to induce IDO expression. Total RNA was isolated from macrophages by using a single-step phenol/chloroform extraction method per the manufacturer's instructions (TRIzol; Life Technologies, Rockville, MD). RNA (1–3 μg) was RT-treated for cDNA synthesis using the Superscript Pre-Amplification System for First Strand cDNA Synthesis (Life Technologies). Equal amounts of samples were used for PCR of cDNAs using primers specific for murine IDO and murine β-actin as control. Murine IDO primers covered nucleotides 45–64 and 1305–1325 (GenBank accession no. M69109) and the sequences were as follows: 5′-AGT TAA CGT GGG GGT CAG TGG AGT AG-3′, sense, and 5′-CGG TTT AAA CTA GTC CTT GAT AGA AGT GGA-3′, antisense. These primers were chosen to include the native ATG transcription start signal as well as the transcription termination signal of the IDO gene and yielded a fragment size of 1.3 kb. Additional nucleotides (underlined above) were included in the primer sequences to create the 5′ end of the primer outside the coding region instriction of the cDNA. Two PCR amplifications were done for each experiment used for adenosine production. Murine β-actin primers were of the following sequence: 5′-ATC CGT AAA GAC CTC TAT GC-3′, sense, and 5′-AAC GCA GCT CAG TAA CAG TC-3′, antisense, and yielded a 287-bp size fragment (21). Each 50-μl PCR reaction mixture contained: 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM each dNTP, 0.2 mM each primer, and 2.5 units Taq polymerase (all PCR reagents were from Applied Biosystems, Foster City, CA). PCR cycling conditions were as follows: denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. A total of 35 cycles were performed. The sizes of amplification products were verified by electrophoresis in a 1% agarose gel using known size markers. For testing successful islet infection with adenosine vectors carrying IDO, we used the same RT-PCR protocol.

Adenosine production. After PCR, the IDO product was cloned in a pbBcs vector (Stratagene, La Jolla, CA) using the Prime PCR Cloner Cloning System (StrataStore, Boulder, CO) and inserted into the pBBI-AdMV-β-gal transfer vector (AEDNO-QUEST Kit; Q-Biogene, Carlsbad, CA), which contains the strong cytomegalovirus 5 (CMV5) promoter driving the expression of the inserted sequence. In addition, this transfer vector contains the coding region for blue fluorescent protein (BFP) under the control of the weaker CMV promoter. Thus, the final transfer vector contains both the DNA of interest and the fluorescent marker BFP. Complete recombinant adenosine vectors (i.e., Ad-IDO and Ad-LacZ) were propagated, amplified, and titrated according to the manufacturer's instructions and results in virus stock concentrations of 2–5 × 10^9 plaque-forming units per milliliter.

Islet infection with adenosine vectors. Murine pancreatic islets were obtained using a method slightly modified from previously published methodologies (22,23). Briefly, islets were isolated from donor pancreases by collagenase digestion followed by separation on a discontinuous Ficoll gradient and purified by handpicking under a stereo microscope. Adenosine infection concentration (multiplicity of infection [MOI]) calculations were based on the generalization that islets contain an average of 1,000 cells (24) and indicates the number of infectious virus particles per cell being treated. Adenosine vectors, at the appropriate MOI, were added in a minimal amount of serum-free media in which the islets were incubated for 1 h at room temperature. Islets were then cultured in complete medium (RPMI supplemented with 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2 mM/l-glutamine, and 50 μmol/l β-mercaptoethanol) for 2 days at 37°C to allow the islets to begin to express the new proteins.

Glucose-stimulated insulin secretion. To measure the ability of the islet cells to produce insulin in response to physiological glucose concentrations, groups of 25–30 adenosine-treated or untreated islets were cultured in 24-well tissue culture plates (Corning, Corning, NY). Islets were first washed in Krebs-Ringer/HEPES buffer (KRH) (25 mM/l-Hepes, 115 mM/l NaCl, 24 mM/l NaHCO3, 5 mM/l KCl, 2.5 mM/l CaCl2, 1 mM/l MgCl2, and 0.1% BSA) containing 3 mM/l glucose. All groups were then incubated with 250 μl KRH containing 3 mM/l glucose for 30 min. The buffer was then replaced with KRH containing 20 mM/l glucose for 30 min and replaced again with KRH with 3 mM/l glucose. All supernatants were collected and analyzed for insulin content by rodent-specific insulin enzyme-linked immunosorbent assay (Crystal Chem, Chicago) using murine insulin as a standard.

T-cell proliferation assay. Proliferation assays were performed to assess T-cell responsiveness in the presence of NOD islet cells, either uninfected or infected with Ad-LacZ or Ad-IDO. Proliferation assays were set up in 96-well flat-bottom tissue culture plates (Corning) with triplicate wells for each experimental condition. Ten thousand trypsin-dissociated islet cells per well were used in each group, with 3 × 10^5 lymph node cells as the responder cells and 2 × 10^7 γ-irradiated spleen cells (3,000 rad, 137cesium, MARK I irradiator; JL Shepherd & Associates, Glendale, CA) as the antigen-presentation cells, also from diabetic NOD mice. Five days after infection for each group, triplicate wells containing media only, islet cells only, and spleen/lymph node cells only. Cells were incubated at 37°C in 200 μl complete RPMI, and replicate plates were harvested on days 5, 6, 7, and 8 of co-culture.

To assess the proliferative state of the cultured cells, 75 μCi [3H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) was added for the final 6 h on the scheduled day. Plates were then frozen, and the entire experiment was harvested and counted at one time. To determine the amount of labeled thymidine incorporated into the cultured cells, plates were thawed, and the content of each well was transferred to glass fiber filter mats (Filtermate 96-well harvester; Packard Instrument, Meriden, CT) and counted on a Top Count microplate scintillation detector (Packard Instrument). Data are presented as counts per minute.

Concavalin A stimulation test. As an additional test of the inhibition of T-cell proliferation, concavalin A (ConA) (Sigma-Aldrich, St. Louis, MO) was found to induce proliferation of five separate IDO-expressing preparations in bulk spleen cell preparations. Adenosine-treated or untreated islets as control were cultured in groups of 400 for 3 weeks. Media from these cultures were saved, tested for tryptophan content, and used as conditioned media for ConA stimulation of spleen cells. Spleen cell suspensions were cultured at 2 × 10^6 cells per well in 96-well plates. Triplicate wells were plated, with and without 5 μg/ml ConA, that contained spleen cells in fresh RPMI or conditioned media from control, Ad-LacZ, and Ad-IDO islets. Proliferation was measured by [3H]thymidine incorporation as described for T-cell proliferation assays.

Fluorometric assay for functional IDO expression. To verify functional IDO expression, tryptophan was measured in culture media by fluorescence spectrophotometry (25,26) after its conversion to norharman by boiling the samples in a trichloroacetic acid, formaldehyde, and FeCl3 solution (Sigma-Aldrich). This transformation resulted in norharman formation from 80 to 90% of the tryptophan present. After cooling, quantitation of norharman was achieved by measuring the absorbance of triplicate aliquots of each reaction mixture at an excitation wavelength of 360 nm with an emission cutoff of 460 nm (Luminescence Spectrometer LS50B; Applied Biosystems). Sample fluorescence values were then compared with a standard curve of known tryptophan concentrations prepared for each experiment.

Microscopic determination of transgene expression. Standard bright field microscopic images were captured by SPOT CCD (charged coupling device) video camera and software (Diagnostic Instruments, Sterling Heights, MI) and prepared for presentation using PhotoShop (Adobe Systems, Klamath Falls, OR). Whole islets in culture were viewed at 100× magnification, with histological sections at 100× or 200× magnification. LacZ expression was detected under bright-field conditions after standard processing for X-gal. BFP was detected by excitation with a mercury lamp and by using a standard commercial DAPI (4′-6-diamidino-2-phenylindole) filter set. IDO expression was visualized using rabbit anti-murine-IDO polyclonal antibody (27), biotinylated donkey anti-rabbit Fab2 (antigen binding fragment 2) (Jackson ImmunoResearch, West Grove, PA), streptavidin/horseradish peroxidase, and AEC (3-amino-9-ethylcarbazole in N,N-dimethyl formamide) chromagen substrate solution (ScyTech Labs, Logan, UT).

Transplantation. Female NODscid mice to be used as transplant recipients were rendered diabetic by a single intraperitoneal injection of 180–250 mg/kg body wt streptozotocin (STZ) (Sigma-Aldrich) freshly dissolved in citrate buffer (pH 4.0). Blood glucose measurements were made 48 h after injection and every day thereafter to assess the diabetic state. Only mice in which blood glucose was >350 mg/dl (Precision QD; Medisense, Coleshill, U.K.) were considered overtly diabetic and used for transplantation. Using this method, recipients were reliably diabetic 2–3 days after STZ treatment.

To prepare isolated islets for transplantation, groups of 400 islets were aspirated into a section of sterile PE-50 tubing (Harvard Apparatus, Holliston, MA) and aggregated by centrifugation. The recipient mouse was anesthetized with Avertin (intraperitoneal injection of 0.30–0.40 mg/gm body wt), and the left kidney was exposed under sterile conditions. A small tear was made at the capsule surrounding the kidney, and the islets were carefully dispensed into the subcapsular space through the tubing connected to a 1-ml syringe. The kidney was then gently replaced and the abdominal wall and skin sutured. Transplant recipients were then monitored for hyperglycemia three times per week to assess graft survival. All recipient blood glucose levels
returned to normal within 24 h of transplantation (data not shown). Graft-dependent maintenance of normoglycemia was demonstrated by nephrectomy of the islet-bearing kidney and subsequent glucose monitoring of the mice. In all cases ($n = 10$, data not shown), the nephrectomized animals presented with hyperglycemia within 2 days of kidney removal.

**Disease transfer.** To test the resistance against autoimmunity of the grafted islets, spleen cells, pooled from a sufficient number of diabetic NOD female mice, were used to transfer the disease to the NODscid recipients after transplantation. Splenocytes were resuspended to a final concentration of $6.67 \times 10^6$ cells/ml. Disease transfer, or ‘challenge’ of transplanted recipients, was performed by a single 0.5 ml i.p. injection of $2 \times 10^6$ cells. As a control for each splenocyte preparation, nonmanipulated NODscid controls were also challenged to verify that viable diabetogenic cells were being injected. Reproducibility of this treatment is demonstrated by this group, which developed hyperglycemia 29.5 ± 5.5 days ($n = 29$, Table 1) after challenge.

**Nephrectomy.** To verify the function of islets transplanted beneath the kidney capsule, some mice were nephrectomized, under avertin-induced anesthesia, and the kidneys containing the transplanted islets were processed for histological analysis. Mice were then monitored daily for the onset of hyperglycemia. Thin sections (10 μm) were cut from the formalin-fixed paraffin-embedded tissues and stained with hematoxylin and eosin for general morphology and with a di-aminobenzidine-labeled anti-insulin antibody (Dako, Carpinteria, CA) for the detection of insulin-producing cells. Islet function was indicated by intense positive staining for insulin, even if there appeared to be infiltrating cells present.

**Statistical analysis.** The Kaplan-Meier analysis for statistical significance was used to compare graft survival curves of the various treatment groups.

## RESULTS

**Adenovirus infection of isolated murine pancreatic islets.** To determine if the adenovirus we generated was able to infect murine islets, isolated islets were incubated with Ad-LacZ or Ad-IDO at a MOI of 50 or 100. After 48 h of culture, viral infection was confirmed after RNA isolation and RT-PCR testing. A band corresponding to the predicted size (1.3 kb) of IDO is seen only in RNA from islets infected with Ad-IDO (Fig. 1, lanes 3 and 4) and not in Ad-LacZ–treated islets (Fig. 1, lane 2).

Adenoviral infection of the islets was also confirmed in vitro by standard methods of X-gal staining of islets that were infected with Ad-LacZ. Resulting dark blue cells are indicative of LacZ production in normally β-galactosidase–negative islet cells. An increase in number of LacZ-positive cells per islet was found with increasing MOI of virus (Fig. 2B–D), and uninfected (MOI of 0) islets showed no evidence of endogenous β-gal activity (Fig. 2A). An MOI of 100 was chosen as the standard for all subsequent experiments on the basis that higher virus concentrations do not appreciably increase staining and result in decreased islet viability (data not shown). Blue fluorescence seen from the BFP encoded by the pQBI-CMV5-BFP vector is also seen with increasing amounts relative to the MOI of virus (Fig. 2E–H). To confirm that the transgene introduced by the viral vectors was coexpressing both BFP and IDO, islets infected in vitro were pelleted, paraffin-embedded, and stained with a polyclonal murine anti-IDO antibody. Detection of positive cells is seen in a similar distribution to that of BFP expression (Fig. 2G and Fig. 3F), indicating coexpression of both proteins from the single adenovirus construct.

**Tryptophan depletion of culture media.** To determine whether the infection of islets resulted in functionally active IDO, groups of isolated islets were used: uninfected (control) or infected with Ad-LacZ or Ad-IDO and cultured for several days. At various time points, media were sampled and assayed spectrofluorometrically for tryptophan concentration by tryptophan conversion to norharman. As shown in Table 2, islets infected with Ad-IDO showed a twofold decrease in the level of tryptophan by day 8 and a 10- to 15-fold decrease by day 14. Uninfected or LacZ-infected islets did not exhibit a significant depletion of tryptophan from the media.

**Insulin secretion from infected islets.** The physiologic functionality of adenovirus-infected islets was tested in vitro by measuring insulin production in response to an increase in glucose concentration. As shown in Table 3, islets expressing either the LacZ or IDO transgenes were able to respond to a glucose challenge in a similar manner as uninfected islets. A four- to fivefold increase in insulin secretion was seen in response to high glucose, with an appropriate decrease of insulin output when the islets

| TABLE 1. Islet transplants expressing IDO result in longer survival time |
|--------------------------|---------------------|---------------------|---------------------|
| Islet treatment          | n                   | Days of graft survival | Median | Mean survival time | SD |
| Control (i.e., no STZ, no transplant) | 29 | 17, 19, 20, 24, 25, 26 (×3), 28 (×4), 29, 28, 30, 31 (×5), 32, 33, 33, 34, 36, 39, 39, 40 | 30.0 | 29.5 | 5.5 |
| Neg (uninfected)         | 9       | 19, 20, 26, 28, 33, 33, 35, 35, 59 | 33.0 | 32.0 | 11.8 |
| Ad-LacZ                  | 10      | 7, 14, 17, 21, 21, 29, 32, 35, 49, 61 | 25.0 | 28.6 | 16.5 |
| Ad-IDO                   | 15      | 24, 30, 32, 37, 37, 40 (×4), 45, 46, 50, 63, 83, 184 | 40.0 | 52.7 | 39.0 |

STZ-treated NODscid recipients were transplanted with untreated or adenovirus-infected islets. Diabetes was adaptively transferred by injection of $2 \times 10^7$ spleen cells pooled from naturally diabetic NOD females. Graft survival times are expressed in days postchallenge. *Ad-IDO transplant survival curves (Kaplan-Meier analysis) were significantly different than control (no transplant, $P < 0.0001$). Neg (transplanted with uninfected islets, $P = 0.01$) and Ad-LacZ–treated grafts ($P = 0.02$). Note that even when removing the longest surviving transplant (184 days), the difference in survival curves remains statistically significant ($P = 0.04$).
FIG. 2. Whole islets infected with increasing MOI of adenovirus reveal increasing numbers of positive cells. Islets were infected with Ad-LacZ (A, MOI 0; B, MOI 10; C, MOI 50; D, MOI 100), detected by X-gal staining or Ad-IDO (E, MOI 0; F, MOI 10; G, MOI 50; H, MOI 100), and cultured in vitro for 48 h. The vector used to produce Ad-IDO contains a coding region for BFP as well. Therefore, blue fluorescing cells have been transfected with adenovirus containing both IDO and BFP. Original magnification ×200.
were returned to a low glucose concentration. On this basis, we concluded that not only is proper insulin regulation maintained by the islets after adenoviral infection, but the production of the virally introduced proteins does not interfere with their physiological function.

In vitro proliferation of diabetic NOD spleen cells exposed to IDO-expressing islet cells. To establish if IDO expressed by infected islets could inhibit T-cell proliferation in vitro, infected islets were dissociated and plated into appropriate wells and cultured for 3 days before the addition of lymph node and irradiated spleen cells from a diabetic NOD mouse. Additional wells were prepared with islets alone and used to test tryptophan levels at the same time points as the thymidine pulse. These wells were plated with either 100 or 200 μl of media. Only wells containing Ad-IDO–infected islets showed a continuous decrease of measured tryptophan (Table 4). The decrease in tryptophan concentration was, as expected, more evident in the 100-μl cultures than in the 200-μl cultures, in which only a partial depletion became evident by day 8. The amount of [3H]thymidine uptake by splenocytes was similar in all treatment groups with the
exception of Ad-IDO islets, allowing a 30% lower proliferation of the culture on day 8 (Fig. 4).

**ConA stimulation of spleen cells.** To further confirm the inhibition of T-cell proliferation was due to depletion of tryptophan, conditioned media were prepared by culturing control and infected islets for 5 weeks. The harvested media were then assayed for tryptophan content and tested in experiments using ConA stimulation of spleen cells in culture. Results of periodic tryptophan measurement are shown in Fig. 5A, where the Ad-IDO treatment of islets resulted in the depletion of tryptophan from the media. At final collection, fresh complete RPMI contained 24.15 nm/ml tryptophan, negative control media from uninfected islets contained 25.83 nm/ml tryptophan, media from LacZ-infected islets contained 23.53 nm/ml tryptophan, and media from IDO-infected islets contained 6.62 nm/ml tryptophan.

**FIG. 4.** Islets expressing IDO can inhibit the proliferation of diabetic spleen cells. Isolated islets were uninfected or infected with Ad-LacZ or Ad-IDO and culture for 72 h. Dispersed islet cells were plated in triplicate (10⁶ cells per well) with or without the addition of 2 × 10⁶ irradiated spleen cells and lymph node (LN) cells from diabetic NOD females. Proliferation was measured by tritiated-thymidine incorporation on days 5 (●), 6 (□), 7 (△), and 8 (■) of the culture. Error bars indicate SD.

Figure 5B shows the proliferation of spleen cells stimulated by ConA after 2 and 3 days of culture. Fresh RPMI supported a much greater amount of proliferation than the conditioned media, possibly because of the removal of nutrients other than tryptophan in the latter as well as the accumulation of waste products. Media from uninfected (Neg) and Ad-LacZ-infected islets resulted in equivalent thymidine uptake, whereas the Ad-IDO conditioned media supported a fourfold lower proliferation. Hardly any increase was seen on day 3 of the Ad-IDO media, whereas the other treated media showed the greatest response to ConA at that time. To verify that the lack of tryptophan was the cause of the reduced response in IDO media, as opposed to accumulation of metabolic waste products, additional wells were cultured with exogenous tryptophan added to the IDO media. The addition of fresh tryptophan was sufficient to sustain cell proliferation that reached control levels (1+T bars in Fig. 5B). Thus, the catabolism of tryptophan by Ad-IDO–treated islets is able to inhibit the proliferation of spleen cells from diabetic NOD mice.

**Transplantation of IDO-transduced islets and diabetes development after adoptive transfer of disease.** In vivo transplantation experiments were performed by injecting collected islets into the subcapsular space of the left kidney of the recipient mice. Initial experiments involved transplantation of isolated and infected islets to syngeneic diabetic recipients using the nonautoimmune mouse strains BALB/c and C57BL/6. All transplants survived until nephrectomy (>90 days) with no evidence of lymphocyte infiltration seen in Ad-LacZ, Ad-IDO, or uninfected islets (data not shown). Nephrectomized animals presented with hyperglycemia within 2 days of graft removal, indicating that the transplants were functional until that moment. Thus, the viral infection procedures do not adversely affect the survival of islet transplants by increasing the immunogenicity of the tissue, at least until the time of nephrectomy (90–100 days after transplantation).

Using the NODscid as the recipient of syngeneic islets and challenging it with diabetogenic NOD spleen cells allows for better experimental control of the autoimmune reaction (28; see also RESEARCH DESIGN AND METHODS). Islets isolated from young (3- to 5-week-old) prediabetic NOD females were infected with Ad-LacZ or Ad-IDO or were used uninfected for control and cultured for 48 h. Islets were then transplanted, in groups of 400, beneath the kidney capsule of STZ-induced diabetic NODscid recipients.

Additional animals were STZ treated; transplanted with Ad-LacZ, Ad-IDO, or uninfected islets; and monitored for hyperglycemia without receiving the adoptive transfer of diabetic NOD spleen cells. All of these recipients remained normoglycemic through >90 days of observation, indicat-

### Table 2

<table>
<thead>
<tr>
<th>Viruses</th>
<th>3 mmol/l glucose</th>
<th>20 mmol/l glucose</th>
<th>3 mmol/l glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neg</td>
<td>295 ± 26.2</td>
<td>1241 ± 26.2</td>
<td>327 ± 23.3</td>
</tr>
<tr>
<td>Ad-LacZ</td>
<td>237 ± 21.9</td>
<td>1201 ± 27.6</td>
<td>260 ± 39.6</td>
</tr>
<tr>
<td>Ad-IDO</td>
<td>288 ± 14.8</td>
<td>1174 ± 42.4</td>
<td>271 ± 26.2</td>
</tr>
</tbody>
</table>

Data are means ± SD. In this experiment, shown as an example, groups of 25 islets were infected with Ad-LacZ and Ad-IDO. Infected islets were compared with uninfected (Neg) islets by culturing them for 4 days before being pulsed with media containing high and low glucose levels. Media were collected after a 30-min exposure to islets. Insulin content measured by ELISA is expressed as picogram insulin per milliliter of media.

### Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3 mmol/l glucose</th>
<th>20 mmol/l glucose</th>
<th>3 mmol/l glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI (control)</td>
<td>16.3 ± 0.8</td>
<td>14.4 ± 0.8</td>
<td>6.7 ± 1.0</td>
</tr>
<tr>
<td>Day 8</td>
<td>13.3 ± 0.5</td>
<td>10.4 ± 0.3</td>
<td>0 (−1.4) ± 0.4</td>
</tr>
<tr>
<td>Day 14</td>
<td>15.2 ± 0.8</td>
<td>10.4 ± 0.3</td>
<td>0 (−1.4) ± 0.4</td>
</tr>
</tbody>
</table>

Data are means ± SD. In this representative experiment, groups of 400 islets were infected with Ad-LacZ or Ad-IDO or used uninfected (Neg) and cultured for 2 weeks. Samples of media were taken at 8 and 14 days for analysis of tryptophan content. Tryptophan is expressed in nanomoles per milliliter.
IDO EXPRESSION EXTENDS ISLET GRAFT SURVIVAL

TABLE 4
Tryptophan levels in a “head start” proliferation assay

<table>
<thead>
<tr>
<th></th>
<th>100 µl media</th>
<th></th>
<th>200 µl media</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neg</td>
<td>Ad-LacZ</td>
<td>Ad-IDO</td>
<td>Neg</td>
</tr>
<tr>
<td>Day 5</td>
<td>14.8 ± 0.3</td>
<td>14.2 ± 1.9</td>
<td>8.7 ± 0.4</td>
<td>19.0 ± 3.1</td>
</tr>
<tr>
<td>Day 6</td>
<td>14.3 ± 1.1</td>
<td>15.7 ± 2.1</td>
<td>10.3 ± 5.6</td>
<td>19.9 ± 2.1</td>
</tr>
<tr>
<td>Day 7</td>
<td>16.2 ± 1.3</td>
<td>16.9 ± 1.1</td>
<td>8.3 ± 2.0</td>
<td>24.7 ± 2.2</td>
</tr>
<tr>
<td>Day 8</td>
<td>16.4 ± 0.2</td>
<td>14.4 ± 0.3</td>
<td>7.0 ± 0.8</td>
<td>16.7 ± 0.6</td>
</tr>
</tbody>
</table>

Data are means ± SD. Islets not cultured with spleen cells were plated in parallel to islets co-cultured with spleen cells to test tryptophan levels of culture media (nmole/ml) on days corresponding to T-cell proliferation measurements in media volumes of 100 and 200 µl per well. Uninfected islets (Neg) were compared with islets infected with Ad-LacZ or Ad-IDO.

FIG. 5. A: Continuous depletion of tryptophan from culture media by Ad-IDO-infected islets. Groups of 500 islets were cultured in 20 ml media for 36 days. Tryptophan content was measured periodically, and media were harvested for conditioned media used in ConA stimulation of splenocytes. ■, Neg (noninfected) islets; ●, media from Ad-LacZ-treated islets; ○, Ad-IDO-treated islets. B: Conditioned culture media from IDO-expressing islets inhibits proliferation of ConA-stimulated spleen cells. Harvested conditioned media prepared in the experiments shown in A were used to culture spleen cells stimulated by ConA. Proliferation was measured by tritiated-thymidine incorporation on days 2 (■) and 3 (■) after addition of 5 µg/ml ConA. I+T, IDO plus tryptophan. Error bars indicate SE of triplicate wells.

maintaining normal glycemias levels solely as a result of the functioning transplanted islets.

Experimental mice were monitored for ~2 weeks after transplantation to ensure proper glucose regulation by the islets and to allow vascularization of the graft. NODscid transplant recipients were then injected with 2 × 10^7 spleen cells from diabetic NOD mice. Recipient blood glucose was monitored three times per week until the onset of diabetes. As a positive control for each adoptive transfer experiment, diabetic spleen cells were injected into NODscid mice that had received neither STZ treatment nor transplant. Destruction of the native islets is demonstrated with the challenged animal becoming hyperglycemic ~30 days after the injection of diabetic spleen cells. These animals were important to include for each batch of spleen preparation to ensure that live diabeticogenic cells were actually being transferred. Survival of transplanted islets could not be attributed to the transgene without verifying that islet-reactive T-cells had been introduced.

Using this model, the survival of transplanted control islets, or islets treated with Ad-LacZ or Ad-IDO, was examined. After accruing 9–15 animals per experimental group, statistical analyses were performed. Table 1 shows the results of the experimental transplants as mean and median survival times of the grafts expressed in days postchallenge with diabetic spleen cells. There was no significant difference between the median survival times of the nontransplanted mice and the animals receiving uninfected or Ad-LacZ islets, whereas the Ad-IDO transplant median survival was 15 days longer than that in the Ad-LacZ treatment group (P = 0.02).

As a result of the prolonged survival after diabeticogenic T-cell challenge, at the time when hyperglycemia became evident, a visibly greater amount of remaining insulin-positive cells were detected in the Ad-IDO transplants when compared with histological characteristics seen in the normal disease progression in uninfected islet grafts of controls (Fig. 3A–D). This could be a result of a slower disease progression in the IDO expression islets maintaining a somewhat greater amount of functioning β-cell mass at the time of death. This result is supported by histology performed on grafted islets in nonchallenged recipients >100 days after transplantation. IDO antibody staining shows several positive cells remaining (Fig. 3E) in IDO-treated grafts. A loss of gene expression with time in vivo is clearly shown when comparing this expression with the staining before transplantation (Fig. 3F). Thus, the expression of IDO after ex vivo adenoviral gene transfer results in prolonged survival in the midst of a diabetic immune...
environment, although the transplants do inevitably succumb to the persistence of the lymphocyte infiltration.

DISCUSSION

Recent results from human trials (6) have indicated that transplantation of isolated human islets is indeed a feasible treatment for human diabetic subjects. Although these adult patients are still receiving some form of immune suppression (tacrolimus, sirolimus, and daclizumab), this study is the first convincing demonstration of normoglycemia attained through the transplantation of islets. Despite the fact that each recipient received islets from more than a single cadaveric donor, the Edmonton study represents an important advance in islet transplantation protocols (6). Efforts to transfer this protocol to children must first involve ways to minimize or completely avoid systemic immunosuppression.

Tryptophan is an amino acid necessary for cellular function and therefore a potential point of regulation of cellular activity. This essential amino acid is used in a variety of biosynthesis pathways, including production of cellular protein and nicotinamide cofactors (NAD, NADP) and the eventual production of neurotransmitters such as serotonin and melatonin. Interestingly, nicotinamide has been shown to protect β-cells from certain toxins and the effects of nitric oxide (31). There is evidence for a role of tryptophan catabolism, by the induction of the specific enzyme IDO, in the immune response of neuronal inflammation due to disease or injury, as well as its involvement in malignancy (9,10), in vitro antimicrobial activity (11–14), and in vitro inhibition of T-cell proliferation (8). More recently, tryptophan depletion has been linked to the maintenance of pregnancy (18).

Combining the known anti-microbial, tumoricidal, and in vitro T-cell inhibition activities of IDO with its in vivo role in pregnancy makes it a candidate with which to protect against recurrence of autoimmunity in the transplant setting. Additionally, IDO could also have an effect in protecting the allogeneic graft against rejection because it appears to protect the semi-allogeneic fetus from maternal T-cell–mediated rejection (18). Local modulation of tryptophan catabolism by IDO may constitute a new potential way to abrogate both autoimmunity and allorejection of islet transplants because both are ultimately mediated by activated T-cells. This inhibition of the activation of T-cells limited to the local environment of the transplant could be an important step in the success of islet transplantation because it would allow the function of the intact systemic immune responses while providing physiological regulation of insulin secretion in the absence of general immune suppression regimens that can be toxic to islets and other organ systems.

The NOD mouse model (19) of human type 1 diabetes provides an excellent tool to test experimental therapeutic protocols that are potentially important to the cure of human disease. The NODscid mouse (20), deficient in mature T- and B-cells, offers a distinct advantage in that disease onset can be more precisely controlled. However, this model also has a distinct disadvantage when compared with the natural disease course of the NOD mouse. By using adoptive transfer of the disease, the transplants in challenged NODscid recipients are being exposed to a sudden onslaught of primed and activated T-cells coming from the spleen of already diabetic mice. In comparison, the progression is much slower in the NOD mouse, in which insulitis becomes evident by 4–5 weeks of age, and hyperglycemia does not appear until 8–12 weeks later (19). Thus, any progress made using this more severe adoptive transfer model (i.e., challenge in the NODscid mouse) may be even more pronounced when tested in the setting of natural disease development of the NOD mouse, although it may more faithfully reproduce the situation created by a therapeutic islet allograft.

The use of replication-defective adenoviral gene delivery vectors has been primarily limited to proof-of-principle type experiments due to several important factors. One is that adenovirus does not integrate into the host genome as do, for example, retroviruses, which require actively dividing cells for viral DNA integration (7). Thus, if mitotic cells are infected with an adenoviral vector, the transfected DNA will be lost after several cell divisions. Islet cells, however, are rather unique in that they have little replicative ability under normal circumstances, so transgene loss due to cell division is not such an important issue.

The second characteristic of adenoviral vectors involves the induction of a strong immune response when the vector is injected directly into the recipient (32,33). The ex vivo methodologies used in our islet transplantation studies do not require direct injection of the virus and also allow for sufficient washing steps before islets are transplanted. However, it has been noted, especially in early experiments with adenoviral vectors, that there can still be a low level of viral protein production found in infected tissues (32,33), which may lead to the immune recognition and clearance of the infected cells. To avoid these adenovirus-specific drawbacks, other viral vectors (e.g., lentivirus, herpes virus, and adeno-associated virus) (34–36) and nonviral protocols (37,38) are being intensively investigated for feasibility of use in islet transplantation studies. As with all experimental procedures, patient safety will be the first and foremost issue to consider once human trials become imminent.

Our experiments with isolated islets transfected with adenoviral vectors confirmed that the physiological function of the β-cells, as measured by glucose-stimulated insulin secretion in vitro and by long-term reversal of the diabetic state in vivo, was not affected by production of the foreign proteins β-galactosidase or IDO. This was important in that even if infected islets were able to completely fend off the autoimmune response, they would be useless to a transplant recipient unless insulin production and regulation remained intact. In addition, in vitro proliferation assays did not detect any increase in immunogenicity of islets as a result of adenovirus infection. This may appear to be in contrast to in vivo studies in the literature where it has been reported that adenovirus-infected cells are cleared from tissues in a relatively short period of time (7,32,33). However, in this setting of ex vivo infection, the isolated islets are washed extensively to remove virus particles before their exposure to T-cells. This can be the reason why a strong response as a result of the virus itself was avoided at least for the length of time that the mouse immune system was exposed to the adenovirus-treated islets. Although the proliferation of spleen
cells in response to islets was measured in vitro after only 5–8 days of culture, in the transplant setting, the grafts are in the presence of immune-competent cells for a much longer time.

As seen with Ad-LacZ, infection by the adenoviral vector containing IDO does not affect the in vitro function of the infected islets with respect to glucose-stimulated insulin secretion. Spleen cells from diabetic donors do not exhibit an increased response to islets expressing IDO when compared with uninfected or Ad-LacZ islets. Also, IDO-infected islets eventually depleted tryptophan from the culture media enough to significantly inhibit the proliferation of diabetic spleen cells once an apparent threshold had been reached, below which additional splenocyte proliferation could not be supported. Islets that had been infected ex vivo and transplanted into STZ-induced diabetic recipients showed a 60% increase in median graft survival time over Ad-LacZ control transplants. The resulting catabolism of tryptophan by the islets was then able to impede their own destruction by the adoptively transferred T-cells.

The fact that IDO expression was able to delay, but not completely prevent, islet destruction could be due to the depletion of enough tryptophan to inhibit proliferation of the infiltrating cells while still allowing these activated cells to initiate the destructive processes. Furthermore, in our model, young prediabetic NOD females were used as islet donors. Infiltration of the islets by T-cells can be seen in these mice as early as 3–4 weeks of age. We were able to purchase animals only after weaning (~3 weeks of age). Thus, it is possible, even though these young animals were all completely healthy and normoglycemic at the time of islet procurement, that there were infiltrating cells already present within the islets. Once transplanted into the immunodeficient NOD.scid recipient, these lymphocytes could be able to survive and proliferate to the point of eventually precipitating disease.

Another possibility to be considered is that IDO expression stops at a certain time, allowing the T-cells to invade the transplanted islets. This prospect, however, could not be easily tested because at the critical time in which the transgene expression should be determined, i.e., at the onset of diabetes, the majority of the islet cells are already dead. However, in support of this possibility, histological analyses of transplants treated with Ad-IDO that survived >100 days in nonchallenged recipients have revealed sparse IDO-positive cells and no visible immune invasion of the grafts. The fact that few IDO-expressing cells remained may be the reason normoglycemia is maintained for a significantly longer duration but eventually does become overcome by the autoimmune destruction. The use of lentivirus or the adeno-associated virus could allow a more complete islet infection with limited immune reactivity and possibly a longer expression time (39).

The introduction of multiple transgenes into one graft is another possible method to aid in survival, either by co-infection of several viruses or by the construction of a vector containing several genes. If tryptophan depletion alone is not sufficient, the addition of anti-apoptotic, antioxidant, or anti-cytotoxic proteins to simultaneously inhibit various pathways of β-cell death may be needed. This is a working hypothesis that is currently being tested in the laboratory based on encouraging results obtained by using the interleukin-1 receptor antagonist protein (40,41), the inhibitor of NFκB (42), and antioxidants such as manganese superoxide dismutase (S.B., A.M.A., M.C., P.D.R., M.T., unpublished data).

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