Comparative Medicine
Copyright 2002
by the American Association for Laboratory Animal Science

Vol 52, No 2
April 2002
Pages 127-135

Isolation, Transplantation, and Functional Studies of Adult Porcine Islets of Langerhans

Thomas Buschmann Nielsen, MD, PhD,* Knud Bonnet Yderstraede, MD, PhD, and Henning Beck-Nielsen, Professor, DrMSc

Transplantation of islets of Langerhans is a possible treatment for type-I diabetes mellitus. However, there is a shortage of donors for such transplantations and the pig may be an alternative source of donor organs. The aims of the study reported here were to establish a method for adult porcine islet isolation that was based on enzymatic digestion using Liberase PI in a semiautomatic set-up, and to evaluate the in vitro and in vivo function of isolated islets. After overnight culture, isolated islets, from five of seven batches, had poor insulin response to an in vitro glucose challenge that was only partially increased by additional challenge with arginine. More than 50% of DNA and 90% of the insulin content was lost during a one-week culture period. With some batch-to-batch variation, in 15 of 25 cases, 4,000 to 7,000 porcine islets cured streptozotocin diabetic nude mice within three weeks following transplantation.

In conclusion, it is possible to isolate viable islets from adult pigs, using a semiautomatic set-up. With batch-to-batch variation, the islets are able to revert diabetes mellitus when transplanted to diabetic nude mice.

Type-I diabetes mellitus is caused by autoimmune destruction of the insulin-producing β-cells in the islets of Langerhans (1). Despite intensive exogenous insulin administration, these patients are at great risk of developing complications from the disease (i.e., angiopathy, neuropathy, retinopathy, and nephropathy), just as intensive insulin treatment increases the risk of hypoglycemic episodes (2). Therefore, replacement of the damaged cells by use of islet transplantation is an attractive alternative to insulin treatment. For more than 20 years, human islet transplantation has been performed with limited success and only 11% of islet transplant recipients have been free of insulin administration one year after transplantation (3). However, recently, the feasibility of human islet transplantation has been confirmed by results reported by Shapiro and co-workers (4). Still, two to three donors are required to cover the needs of one recipient, which underlines the shortage of human donors for islet transplantations. Because its physiology is similar to human physiology, the pig has drawn much attention in the search for alternative donors for xenotransplantations. Furthermore, pigs can be bred under gnotobiotic conditions, they breed fast, have large litters, and can be bred under standardized conditions making it possible to optimize important conditions, such as age, body weight, strain, and rearing. Using pigs for human organ transplantation has come a large step closer by the production of transgenic pigs and the recent cloning of a pig (5). However, adult porcine islets are surrounded by a thin and incomplete peri-insular connective tissue capsule (6-8) making them fragile to stressful handling during isolation procedures.

More than 10 years ago, Ricordi and co-workers (9) described a semiautomatic method of porcine islet isolation, and even though numerous modifications of that method have been applied by various groups over the years, porcine islet isolation remains a delicate process with inconsistent outcome. Numerous factors can influence the outcome of adult porcine islets isolation: donor strain (10-13), age (11, 14, 15), rearing (16), organ procurement (16-18), warm and cold ischemia time (19), storage solution, organ distention, enzymes (purity, batch, and concentrations) (20-22), pH during isolation (11), and purification technique and gradients (23, 24).

The aims of the study reported here were to establish a method for adult porcine islet isolation and to evaluate the function of isolated islets in vitro by perifusion and in vivo after transplantation to diabetic athymic mice.

Materials and Methods

Animals. Hybrid pigs, a cross between Danish Landrace and Yorkshire breeds, were used as donors. Pigs were purchased from a local farmer (Pilegaard, Odense, Denmark). The pigs were routinely tested for: *Hæmatopinus suis*, *Sarcoptes scabiae* var. *suis*, *Pasteurella multocida*, *Brachyspira hyodysenteria*, *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae* and porcine respiratory and reproduction syndrome.

Mice (Balb/cA Bom-nu, M&B, Ry, Denmark) were used for transplantations (health status of the mice was the same as it is today and can be found at www.m-b.dk). The mice were kept in type-II cages (267 × 207 × 140-mm, Scanbur, Lille Skensved, Denmark), one mouse per cage, under a hood with 50 to 70 filtered air change/h (Type D-110 Scantainer, Scanbur). Cages were disinfected with 70% ethanol before use. Temperature in the room was kept at 23°C, and humidity was 55%. The animals had ad libitum access to autoclaved tap water (and drinking bottles) and radiated pelleted food (Altromin, Brogaarden, Denmark). When handling the mice, single-use coats, caps, surgical masks, and single-use rubber gloves were worn. Gloves were

Received: 9/07/01. Revision requested: 11/05/01. Accepted: 1/16/02.
Department of Medical Endocrinology/Biomedical Laboratory, Odense University Hospital, Sdr. Boulevard 29, DK-5000 Odense, Denmark.
**Corresponding author.*

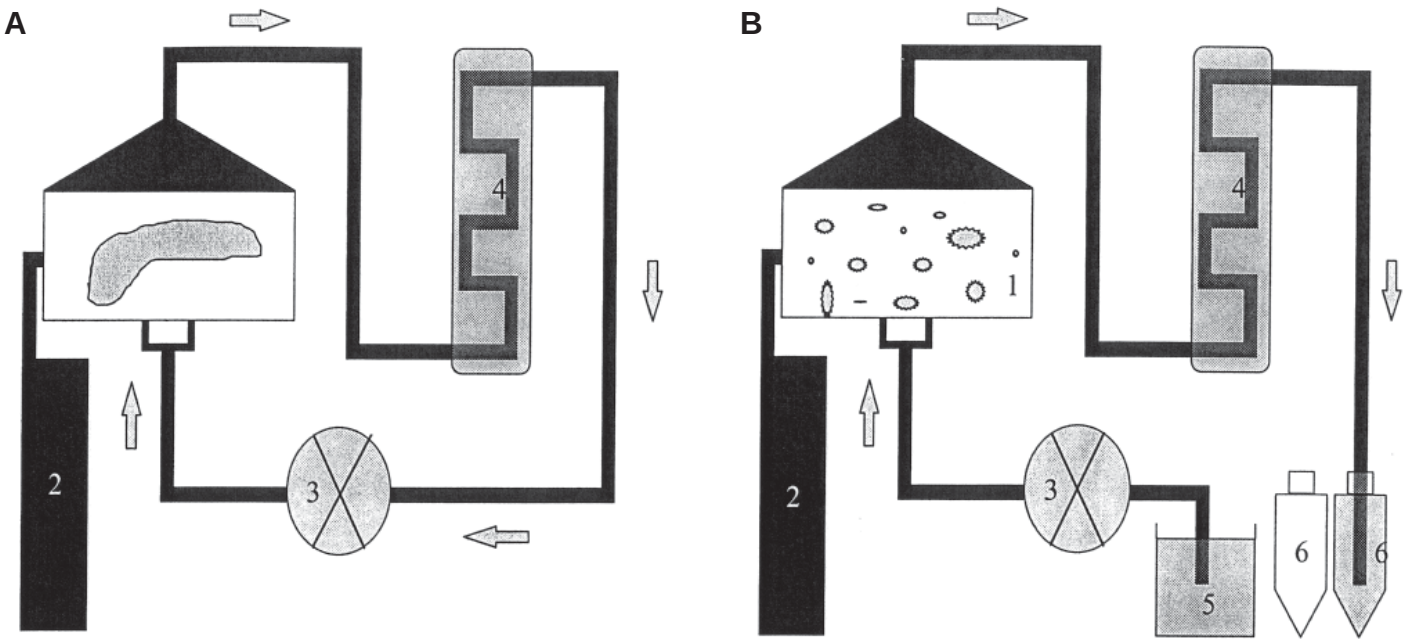

Figure 1. Schematic representation of the semiautomatic system used for adult porcine islet isolation. For digestion (A), the distended pancreas is placed in the digestion chamber (1), which is connected to a vertical shaker (2). Collagenase solution is recirculated through the digestion chamber, using a peristaltic pump (3). Temperature is controlled by pumping the collagenase solution through a heat-exchange column (4), which is connected to warm and cold water supplies. For dilution (B), the system is opened, and Hanks' balanced salt solution (HBSS) is drawn in one end (5), pumped through the digestion chamber, cooled at the heat-exchange column, and collected in 250-ml centrifuge tubes (6).

disinfected with 70% ethanol between handling of individual mice. All experiments were approved by the Danish Animal Experiments Inspectorate.

Isolation of islets. A total of 61 pancreata were processed. Twenty isolations were performed to establish the semiautomatic set-up and improve on technical skills and equipment. The next 31 isolations were performed to optimize organ procurement (local versus slaughterhouse), enzymes (Liberase PI versus Liberase HI [Boehringer-Mannheim, Mannheim, Germany]), and the extent of mechanical disintegration (shaking). On the basis of these findings we decided on the following procedure for the subsequent 10 isolations.

Organ procurement. The yield from porcine islet isolations have been documented to increase with increasing age of the donor (11, 14, 15, 17). Therefore, retired breeders, two years old and weighing 175 to 250 kg, were used as donors for our experiments. To minimize cold ischemia time (from the cooling of the organ till processing), pigs were delivered to our animal care facilities in the morning. Within half an hour they were given an intramuscular injection of ketamine (10 mg/kg of body weight; Pfizer, New York, N.Y.). Ketamine was given to sedate the animals before they were euthanized by applying a penetrating captive bolt to their forehead. Ketamine was chosen, because it is not known to reduce convulsions as are barbiturates. Convulsions after euthanasia are thought to promote bleeding of the animal, which is important, because residual blood seems to inhibit collagenase activity (18). Euthanasia was performed according to European Union (EU) law.

When the pig had been euthanized, it was immediately incised in the carotid artery and left bleeding for two to five minutes until the end of convulsions. Thereafter, the pig was lifted head up, incised once more in the iliac artery to continue bleeding, and operation procedures were initiated. Laparotomy was performed. The gastrointestinal tract was cut at the esophagus and the stomach, and the intestines were removed en block. The splenic lobe of the pancreas was located, and the pancreas was carefully dissected free from the surrounding tissues. To minimize the risk of infection and to decrease organ temperature, the pancreas was washed twice in ice-cold isotonic saline (2 × 500 ml) (25), and immediately thereafter, placed into 250 ml of 4°C University of Wisconsin (UW) organ preservation solution (Viaspan, Dupont Pharmaceuticals, Hertfordshire, England), which was then put on ice (26). Warm ischemia time (from the time of cardiac arrest to cooling of the organ) ranged from 5 to 10 min.

Digestion. While it was laying in a tray placed on ice, the pancreas was further dissected free from connective tissue and lymph nodes. Due to the fact that islets are more abundant in the corpus and caudal regions of the pancreas (27), only these parts were used for islet isolation. The pancreatic duct was located and cannulated, using a 18-gauge catheter. The catheter was fixed to the organ by a pair of forceps. Following cannulation, the pancreas was distended with cold (4°C) Liberase solution by manual injection over five minutes, using a 50-ml syringe. Liberase solution was made by dissolving the enzymes in UW solution. Distention with cold enzymes was performed within 30 min after organ retrieval because distension beyond this time cannot be expected (19). Liberase PI (Batch No. 49085920, Boehringer Mannheim, Mannheim, Germany) was used at a concentration of 2.3 mg/ml.

While still in 4°C UW solution, the distended organ was brought to the islet isolation laboratory and placed in a digestion chamber. From this point on, procedures were performed aseptically. A semi-automatic set-up, originally described by Ricordi and co-workers (9) was used: the chamber had two inlet ports, one outlet port, and a port for temperature control. The

chamber was placed on a shaker with vertical oscillations and adjustable amplitude (Fig. 1; set for 22 mm) and frequency.

The system was filled with an additional 500 ml of room temperature Hanks' balanced salt solution (HBSS) supplemented with 100 U of penicillin and 0.1 mg of streptomycin/ml, and 10 m*M* HEPES buffer (all from Gibco, Life Technologies, Paisley, Scotland). A peristaltic pump was used for recirculating the enzyme solution at the rate of 200 ml/min in an initially closed loop. Enzymes passed a heat exchange column and were pumped through the digestion chamber. Control of the temperature was obtained by switching between a warm (34°C) and a cold (4°C) water supply to the heat exchange column. For the first 25 min, temperature was increased by 1°C/min until a temperature of 28°C was reached. During this phase, the digestion chamber was gently shaken at the rate of 100 oscillations/min. After 25 min, shaking was increased to 200 oscillations/min.

From the enzyme solution leaving the digestion chamber, one-milliliter samples were taken at frequent intervals (every minute when tissue appeared in the solution), stained with dithizone, and examined under the microscope to evaluate progress in the digestion process. During the entire procedure, pH was monitored and kept at a constant value between 7.3 and 7.4 (11) by addition, when necessary, of 1*M* NaOH to the solution.

Dilution. When a number of 5 to 10 islets free of exocrine tissue appeared in a sample, the digestion process was stopped by opening the system and flushing the digestion chamber with HBSS at room temperature, thereby slowing down the enzymatic process by cooling and diluting the enzymes. The heat exchange column was switched over to the cooling circuit (4°C), thereby further cooling the effluent from the digestion chamber, and the digest was collected in 250-ml centrifuge tubes (Corning, Rochester, N.Y.) placed on ice. To further slow down the digestion process, each of these tubes contained 25 ml of newborn calf serum (NCS, Gibco, Life Technologies, Paisley, Scotland). The digest was centrifuged at 80 ×*g*, 4°C, and washed twice in 4°C HBSS. Samples were taken to evaluate the number of islets before purification, and the tissue was left resting in 4°C UW solution for 45 min. Prepurification storage in UW solution minimizes swelling of the islets, thereby facilitating islet purification (28).

Purification. Islets were washed once more at 80 ×*g*, re-suspended in 100 ml of UW solution, and further dissolved in 400 ml of Ficoll (Biochrom, Berlin, Germany), with density of 1,082 g/L, which was made by dissolving Ficoll (1,090 g/L) in 10% HBSS, 1 ml of 1*M* NaOH, and 20 m*M* HEPES (21). The islet/Ficoll suspension was loaded into a 500-ml blood bag (Baxter Healthcare, Allerød, Denmark), and from there into a Cobe 2991 cell separator (Cobe Laboratories, Lakewood, Colo.) (24, 29). During centrifugation at 800 ×*g*, a top layer of 150 ml of culture medium M199 (Gibco, Life Technologies, Paisley, Scotland) with 10% NCS was loaded by use of a peristaltic pump (speed, 60 ml/min). The Cobe cell separator was run for 5 min, then the content was unloaded into 50-ml centrifuge tubes containing 25 ml of M199 plus 10% NCS. Samples were taken from each tube, stained with dithizone, and evaluated under the microscope for the content of islets. Fractions containing > 80% pure islets were collected, washed twice at 200 and 80 ×*g* in M199 plus 10% NCS. Samples were taken in duplicate to evaluate islet yield and final purity after purification.

Culture. Adult porcine islets were incubated in humidified air (37°C, 5% CO_2/95% atmospheric air), and cultured in 140-mm petri dishes containing RPMI 1640 medium supplemented with 20% porcine serum, 2 m*M* L-glutamine, 10 m*M* HEPES, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 μg/ml; all Gibco) (21).

Induction of diabetes mellitus. Streptozotocin (Sigma Chemical Co., Steinheim, Germany) was freshly dissolved in citrate buffer, pH 4.5. Mice were made diabetic by a single injection of this compound (275 mg/kg) into a tail vein. Some mice did not respond sufficiently to this dosage and needed another injection of streptozotocin (200 mg/kg) before diabetes mellitus was induced. Only animals with at least two random measurements of blood glucose (BG) concentration > 20 mmol/L were used as recipients. After confirmation of diabetes mellitus, mice were treated with insulin until used for transplantation.

Transplantation surgical procedures. Mice were anesthetized by subcutaneous administration (0.065 ml/10 g) of a mixture made from 0.25 ml of Diazepam (5 mg/ml; Dumex Alpharma, Copenhagen, Denmark) dissolved in 0.5 ml of isotonic sodium chloride and 0.25 ml of Hypnorm (Fentanyl citrate [0.315 mg/ml] and Fluanisone [10 mg/ml], Janssen Cilag, Copenhagen, Denmark) dissolved in 0.5 ml of isotonic sodium chloride as well. Surgical procedures were carried out under an operating microscope (OPMDI 70, Zeiss, Germany). Aseptic surgical technique was used. Access to the left kidney was obtained via lumbar incision, capsulotomy was performed, and a small amount of air was injected under the kidney capsule, using a 25-gauge venflon needle attached to a 1-ml syringe. To avoid shrinking of the kidney capsule, it was kept moist with isotonic NaCl during the procedure. Using a microsyringe, islet tissue was aspirated into a polythene tube (ID, 0.58 mm; Portex, Hythe, England), centrifuged, and carefully injected under the kidney capsule by placing the tip of the polytene tube into the cavity created under the kidney capsule. After removing the tube, the capsule was cauterized. The kidney was carefully replaced, and the incision was sutured in two layers. After surgery,, the mice were given a subcutaneous injection of Temgesic (0.075 mg/kg; Buprenorphine, Reckitt & Colman, Hull, England).

Follow-up evaluation. After transplantation, BG concentration was measured, using a Medisense glucometer (Medisense, Snedsted, Denmark), on five to 10 μl of tail vein blood once daily for the first two to three days, then one or more subcutaneous injections of glucose was administered if necessary to avoid hypoglycemia. Thereafter, BG concentration was measured once weekly.

Glucose tolerance testing. After normoglycemia had been achieved for at least two weeks, glucose tolerance testing was performed: after food had been withheld for two hours, glucose (3 mg/kg, in a 50% solution) was administered either intragastrically through a gastric tube or intraperitoneally by injection. The BG concentration was measured 10, 15, 30, 60, and 120 min later. Between the two tests, at least a 48-h period of resting was allowed.

Nephrectomy. Anesthesia was performed as described previously. Access to the kidney was made via lumbar incision, and the kidney was carefully dissected free from the surrounding tissue. The kidney stalk was ligated and the incision was sutured in two layers.

Counting of islets. Samples collected from islets in suspension were counted in doublets. The samples were stained with

dithizone, which stains zinc in insulin granules and gives a reddish appearance. Islets were sized, using a calibrated grid in a phase contrast microscope, and were converted into islet equivalents (IEQ), with average diameter of 150 μm (30). Islets smaller than 50 μm were not counted.

Perifusion. Basically, two systems have been applied to evaluate the in vitro insulin secretory capacity of isolated islets: static incubation and dynamic perifusion. Static incubation has the advantage of being simple and cheap to perform. Perifusion is believed to yield more dynamic description of islet function, possibly better mimicking the in vivo function. Perifusion was carried out by use of a Brandell Suprafusion 2000 system (Brandell, Gaithersburg, Md.). Reagent was driven by a peristaltic pump (flow rate 0.5 ml/min) through six parallel chambers. In these chambers, islet tissue was placed in a supportive gel (Biogel P4, BioRad, Hercules, Calif.) between two filters. The effluent was collected in two-milliliter tubes (Sarstedt, Nümbrecht, Germany) placed in a rack on an ice tray. A computer allowed programmable reagent and sample collection. A heating system and a plastic jacket provided an environment with constant temperature (37°C).

As reagent, we used a Krebs Ringer HEPES buffer stored at 4°C and freshly supplemented with the relevant amount of glucose and arginine before use. To obtain stable baseline insulin secretion (31, 32), islets were initially perfused for 60 min with 2.5 m*M* glucose, followed by stimulation with 16.7 m*M* glucose, with or without 10 m*M* arginine, for the next 60 min, and finally with 2.5 m*M* glucose for the last 60 min. Samples were collected at the end of the stabilization period, then every 2.5 min for the first 10 min of stimulation, and every 10 min thereafter. Samples were frozen at -20°C until analyzed for insulin content.

Insulin analysis. When total insulin content was measured, samples were taken in duplicate from tissue in suspension. One milliliter of glycine-bovine serum albumin (BSA, 2.5%) buffer (pH 8.8) was added to the samples, and they were sonicated twice for 15 sec.. Samples were centrifuged at 800 ×*g* 10 min, and supernatants were collected and stored at -20°C until analyzed for insulin concentration.

Insulin was measured by use of a double-antibody technique. The assay uses a time- resolved immunofluorometric signal from Europium (33) (Delfia, Wallac, Denmark), and a porcine insulin standard was used (porcine actrapid, a generous gift from Novo Nordisk, Bagsværd, Denmark). Measurements of total insulin content were determined in dilutions of 1:50 and 1:100.

Measurement of DNA content. The DNA content was measured in duplicate, using a Picogreen dsDNA quantification kit (Molecular Probes, Leiden, The Netherlands). Calf thymus DNA was used as standard. Fluorescence was measured by use of a fluorometer set at wavelength of 480 nm.

Results

Islet yields. A consecutive series of 10 adult porcine islet isolations was performed with constant basic settings. In one instance, the pre-purification yield was too poor to proceed to purification (25,000 IEQ), and in two instances, the purification process failed and resulted in almost unpurified islets. Islet isolation and purification were, therefore, completed in only seven instances. All in all we had a pre-purification yield of 116,000 ± 41,000 IEQ (median ± 95% CI, n = 10), range 25,000 to 221,000 IEQ, and post purification yield of 57,000 ± 28,700 IEQ (median ± 95% CI, n = 9), range 0 to 110,000 IEQ. Before purification, IEQ amounted to 1,068 ± 387/g of pancreatic tissue, and 457 ± 306/g of pancreatic tissue (median ± 95% CI) after purification. The degree of purification was estimated to 80 to 90%. Sixty nine ± six per cent (mean, ± 95% CI) of the islets measured between 50 to 100 μm, 22 ± 6% between 100 and 150 μm, 7 ± 2% between 150 and 200 μm, and only 1 ± 1% were larger than 200 μm. Postpurification islet size was similar: 69, 24, 6, and 1%, respectively.

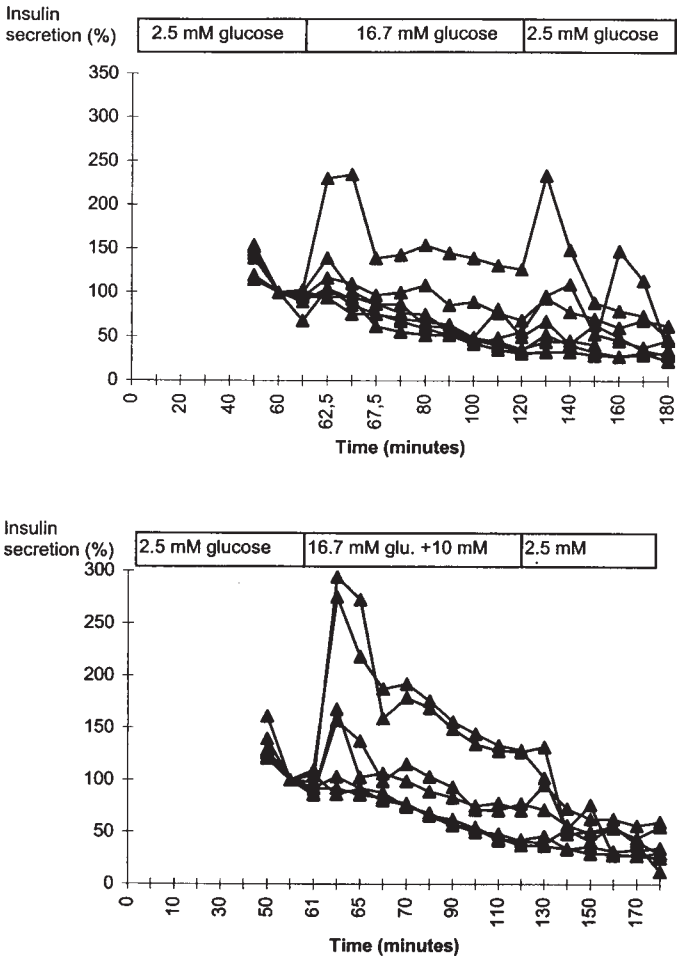

Figure 2. Perifusion of adult porcine islets. Islets were initially perifused for 60 min with buffer containing 2.5 m*M* glucose before being challenged with 16.7 m*M* glucose (upper panel), with or without 10 m*M* arginine (lower panel), for 60 min, and finally with 2.5 m*M* glucose for 60 min. Data are expressed as percentage of insulin secretion immediately before stimulation (at time = 60 min, insulin secretion = 100%).

Perifusion. When islets were cultured for one night and perfused as described, for five of seven isolations, we found transient increase in insulin secretion when they were stimulated with 16.7 m*M* glucose (Fig. 2). The initial insulin secretory peak was followed by a continuous decrease in insulin secretion for the remaining stimulation period. A second-phase insulin response was not observed. At cessation of stimulation, a significant increase in insulin secretion was observed, followed by a gradual decrease. When challenged with additional 10 m*M* arginine, in four of the seven isolations, there was a transient increase in insulin secretion (Fig. 2). On cessation of stimulation, a decrease in insulin secretion was not observed until 20 min

later. Looking at the islet batches responding to a glucose challenge, four of the five had an increased insulin secretory response to the glucose plus arginine challenge (median, 110%). One batch only responded to the glucose challenge, but not to the glucose plus arginine challenge.

To evaluate the impact of culture, adult porcine islets were cultured for six days. On the first day of culture, > 50% of the initial amount of DNA and insulin was lost. The rapid decrease in insulin content continued, whereas a more moderate decrease in DNA content was observed. Indeed, looking at the amount of insulin per DNA, a continued decrease also was observed (Fig. 3).

To evaluate the influence of culture on the in vitro function, perifusion was performed in two instances after one week of culture. The islets remained responsive to a glucose challenge. However, compared with the primary glucose challenge, one responded with increased insulin secretion and the other with reduced insulin secretion (Fig. 4).

Transplantations. Twenty-five diabetic Balb/c nu/nu mice were transplanted with 4,000 to 7,000 IEQ beneath the renal capsule. Fifteen mice became normoglycemic within 3 ± 1 weeks (median ± 95% CI) with range of one day to eight weeks. For unknown reasons, three mice died in the perioperative period (within 24 h). Seven mice remained hyperglycemic within an observation period of three months, although one of these mice had a low glycemic value of 11 mmol/L.

All mice (N = 7) with hyperglycemia for > 3 months were transplanted with islets originating from only two isolations. Mice transplanted with islets from these two isolations never became normoglycemic. Of the 15 mice that became normoglycemia, eight were transplanted with 4,000 to 5,000 islets and became normoglycemic within an average of 2.1 weeks and seven were transplanted with 6,000 to 7,000 islets and became normoglycemic within 3.4 weeks (not significant on the basis of the Wilcoxon rank sum test).

Due to technical difficulties, graft removal by nephrectomy was successfully completed in only five of the 15 mice with normalized BG concentration. After nephrectomy, hyperglycemia returned in four mice, but one mouse remained normoglycemic. However, in the latter, normoglycemia had been achieved immediately after transplantation, indicating at least transient function of the graft.

Glucose tolerance testing. Glucose tolerance testing was were performed on mice becoming normoglycemic after transplantation. Transplanted mice had an average preprandial BG concentration of 3.3 ± 0.7 m*M* (median ± CI), whereas controls had an average BG *concentration* of 5.6 ± 1 m*M* (median ± CI). After glucose administration, BG concentration increased to the same value in the two groups. However, the transplanted mice cleared the glucose load faster than did controls (Fig. 5). Both groups had two-hour values at or lower than initial preprandial BG concentration.

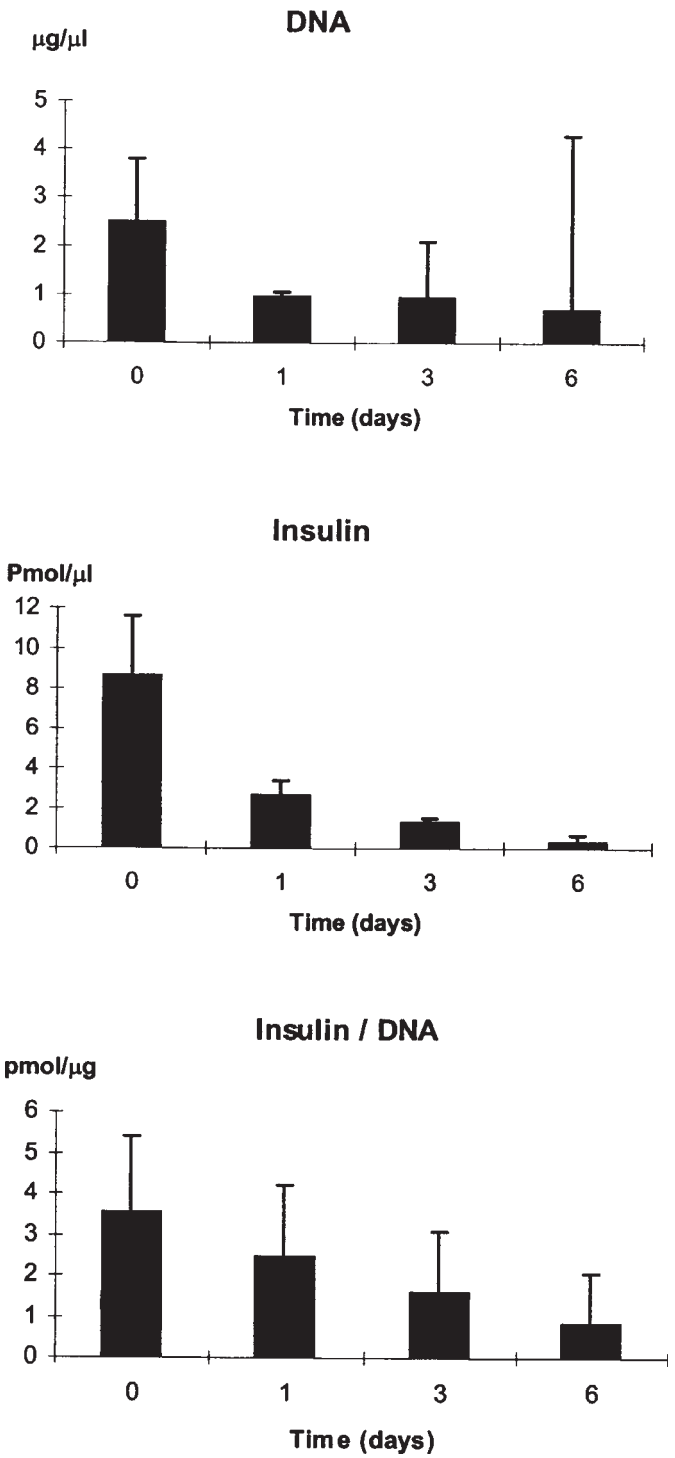

Figure 3. The DNA and insulin contents of adult porcine islets cultured for six days. ($P < 0.05$, Friedman's two-way analysis of variance), Data are expressed as median ± 95% CI (N = 4).

Discussion

Because of the potentially large number of islets to be obtained from adult pigs (12), it is an attractive source of islets for xenotransplantation. More groups have focused on the isolation procedure, but results vary considerably (9, 11, 14, 17, 19-21, 34-38), though most groups use basically the same semiautomatic method as that described by Ricordi (9). It is well recognized that isolation of porcine islets is a delicate process, with huge variability in results (39). Still, comparing our results with those reported by others on porcine islet isolation, low yields with an average of 450 IEQ/g of pancreatic tissue were achieved. Factors influencing the outcome of islet isolations are numerous. One possible reason for our lower yields could be our choice of donors: compared with dog, rat, and human islets,

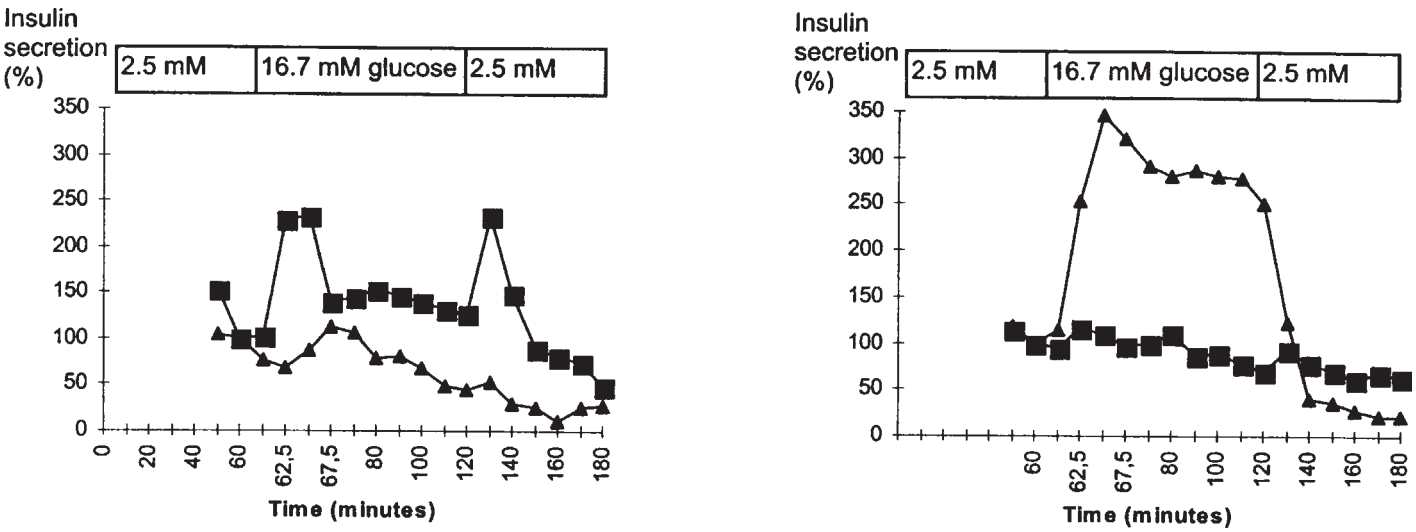

Figure 4. Perifusion of adult porcine islets after overnight culture (boxes) and culture for six days (triangles) respectively. Islets were initially perfused for 60 min with buffer containing 2.5 m*M* glucose before being challenged with 16.7 m*M* glucose, and finally 2.5 m*M* for 60 min. Each panel represents one batch of islets.

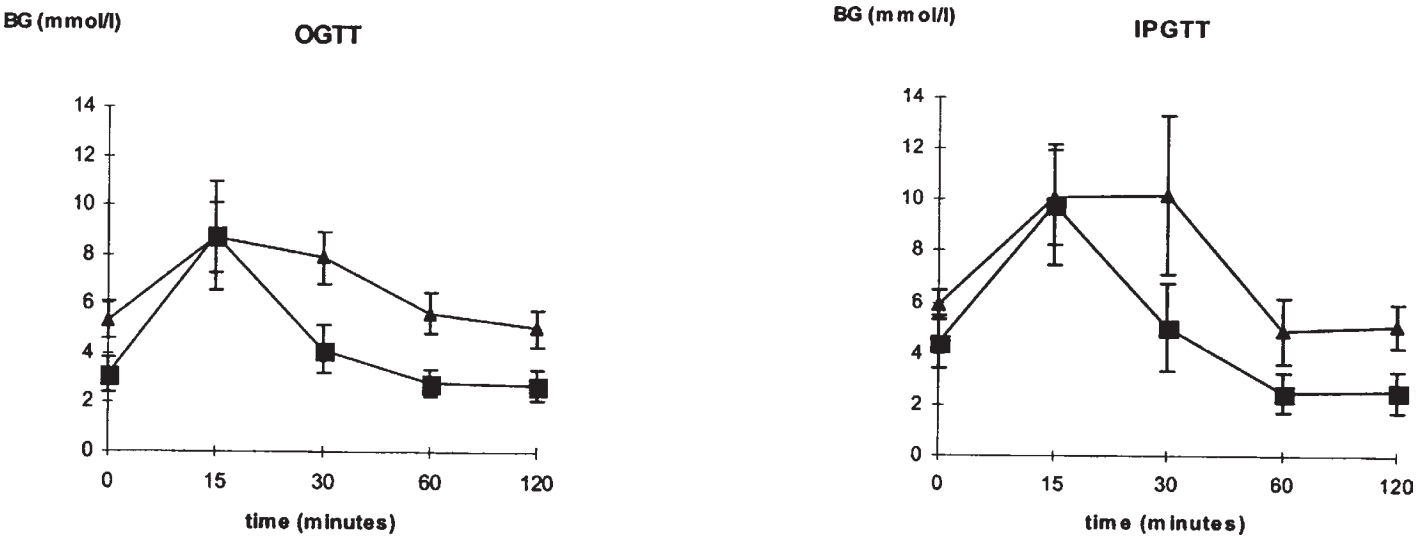

Figure 5. Glucose tolerance tests performed on mice that were transplanted with adult porcine islets and consequently achieved normoglycemia. Transplanted mice (N = 14, boxes) are compared with a group of non-diabetic controls (N = 9, triangles) for oral glucose tolerance test (OGTT) results (left panel) as well as intraperitoneal glucose tolerance test (IPGTT) results (right panel). Data are presented as median ± 95% CI. Difference between groups is significant ($P < 0.05$, Wilcoxon rank sum test) at time 0, and 60 and 120 min. BG = blood glucose (concentration).

adult porcine islets are surrounded by a thin peri-insular capsule (6, 7) that is easily destroyed during enzymatic isolation procedures.

Immunohistochemical analysis of pancreata from various strains of pigs has revealed different compositions and amounts of collagen around the islets (7, 10, 12), possibly making islets in some strains more resistant to mechanical and enzymatic disintegration. Furthermore, some strains have more and larger islets than do others (7, 10). Indeed, differences in yield from different strains has been reported (7, 11). Donor age also influences results. The composition of the peri-insular capsule changes with age (8, 17), and larger yields can be obtained from older pigs/retired breeders (11, 14, 15). We used two-year-old hybrid pigs, a cross between Yorkshire and Danish Landrace breeds; to our knowledge, there are no previous reports on islet isolation from this specific combination of strains. Even though donors came from the same supplier, genetic differences between donors is inevitable, which may affect reproducibility of the isolations (37). Even in pigs from the same litter there may be huge variability in the number of islets (7).

Compared with others, we had relatively low warm and cold ischemia times, possibly reducing the risk of pre-isolation injury to the tissue (19, 40).

The most commonly used product for islet isolation is a blend of enzymes included in collagenase. We used a new enzyme blend, Liberase PI, which is a purified enzyme blend (from collagenase) specifically designed for isolation of porcine islets (20). The choice of enzyme is of great importance as the older collagenases were a poorly defined mixture of several enzymes (41), giving unpredictable large variability in activity from

batch to batch, and activity loss during prolonged storage (22). Liberase is a purified enzyme blend consisting of well defined enzymes (20), thereby reducing batch-to-batch variability. However, there are still reports of up to 50% variability in enzyme activity (21). Recently, excellent results from use of Liberase HI (designed for human islet isolation) for adult porcine islet isolation have been reported (21). However, we had only occasional success using similar enzymes and isolation set-up. Liberase PI and Liberase HI have been tested against conventional crude collagenase (20-22) and were proven to result in better yields, but comparison between the two Liberases has not yet been published to our knowledge.

One of the most crucial points of semiautomatic islet isolation is when to stop the digestion process (9, 14) and start diluting the system. The process has to be stopped at a moment when a sufficient number of islets free of exocrine tissue appears in the samples taken. If this point is missed, the result will be overdigestion and defragmentation of the islets. If the process is stopped too soon, it will result in islets trapped in exocrine tissue, thus deteriorating the purification process. In our experience, the interval from liberation of free islets to commencement of overdigestion is short (1 to 2 min) and may easily be missed. Others have been able to work with constant digestion time (34, 36); however, even though we kept our isolation conditions as constant as possible, digestion time varied from 35 to 60 (median, 45) min, and an accurate prediction of digestion times could not be made. Apart from donor characteristics, small differences in parameters, such as organ bleeding, ischemia time, rate of temperature increase and weight of the organs, may all add up and cause the variability in the digestion time.

Purification of islets after isolation is necessary to minimize the amount of tissue to be transplanted and to remove exocrine tissue, which contains enzymes potentially damaging to the islets (16). Also, exocrine tissue may be more immunogenic than are pure islets (23). Islet recovery after purification is dependent on difference in densities of islet and exocrine tissue, and this difference may diminish because of edema of the tissue (23). Pre-purification treatment of the tissue, including isolation procedure, is, therefore, of great importance (28). The method used for purification also is of great importance (23). We had an average of 48% islet recovery after purification, using a discontinuous monolayer gradient on a Cobe cell separator (21, 24). This is not satisfactory but comparable to what is reported by others for porcine islet purification (9, 35, 37) as well as human islet purification (29, 42). Unfortunately, many groups fail to report pre- and postpurification yields (14, 17, 20, 21, 38).

When the in vitro function is evaluated by challenge with glucose during static incubation, reports on stimulation index (insulin secretion during challenge divided by insulin secretion before challenge) for adult porcine islets ranges from 1 to 3.6 (17, 21, 34, 35, 43-45). When perifusion is used, reports on insulin secretory response toward various secretagogues also varies considerably. Warnock and co-workers (37) reported lack of glucose-stimulated insulin response after overnight culture. However, others (19, 46-48) obtained stimulated insulin responses with stimulation index from 2.2 (47) to 9.0 (19). After overnight culture, we found a small response to a glucose challenge alone (range, 5 to 229%) in five of seven islet batches. Apart from culture conditions, integrity of the islet may explain the diverging results. Depending on isolation and purification procedures, islets may be more or less traumatized during this process, leading to temporary loss of glucose responsiveness (49). A large loss of the peripherally located glucagon-secreting α-cells, during islet isolation procedures, may damage the paracrine function manifested by these cells on the β-cell insulin secretory response (50). Culture may restore islet function, and clear the islet population from totally damaged islets (43). Indeed, a large number of islets are lost during the first days of culture (20, 37).

In the two instances when we challenged islets with glucose after one week of culture, the results were somewhat diverging. When we challenged the islets with additional arginine, the insulin secretory response was increased in four of the five batches responding to a glucose challenge (range, 57 to 194%). Others reported significant insulin secretory responses to arginine challenge alone (34, 51). Arginine is supposed to potentiate glucose-induced insulin secretion (52).

The optimal way to evaluate islet function is in vivo (53), and when we transplanted islets underneath the renal capsule of streptozotocin-induced diabetic athymic mice, normoglycemia could be restored in some but not all animals. The variable in vivo function was related to the specific isolation because all of the failed grafts came from only two isolations. Even though large numbers of islets were transplanted, it took an average of three weeks for normoglycemia to be restored. This is comparable to what is reported by some (54, 55), whereas others reported almost immediate normalization of BG concentration after transplantation of 1,500 to 2,000 porcine islets to diabetic nude mice (19, 35, 48). In our study, difference in the time needed to normalize BG concentration was not apparent between those transplanted with 4,000 to 5,000 islets, compared with those transplanted with 6,000 to 7,000, which may indicate that viability rather than number of islets was the limiting factor. Indeed, some isolations were faster than others in restoring normoglycemia, underlining the difference in viability/function from isolation to isolation. It is not clear why some grafts required three to four weeks to obtain complete function. It could be that neovascularization, which takes place within two to 14 days (56, 57), was necessary for the grafts to function. Beger and co-workers (58) reported that, after transplantation, pseudo-islets (islets generated from single cells clustering into islet-like structures during culture) had delayed revascularization, compared with normal intact islets. If disintegrated islets have to reorganize into pseudo-islets after transplantation, they might have somewhat delayed revascularization. This could explain why some isolations have function almost immediately, whereas others—containing more disintegrated islets—need more time to gain complete function. Looking at individual isolations, nothing could be transferred from the observed in vitro function to the in vivo function, which is comparable to what is generally believed (53).

In conclusion, our results indicate that, despite numerous reports of refinement of the porcine islet isolation procedure, there is continued difficulty in establishing a safe and reproducible method for isolation and purification of adult porcine islets of Langerhans. After overnight culture, adult porcine islets responded poorly to a glucose challenge, though this response was somewhat augmented by additional challenge with arginine. However, it is possible to produce viable adult porcine islets that can achieve adequate function in vivo, after transplantation into a xenogeneic model.

Acknowledgments

The skillful assistance of technician Hanne Thayssen is gratefully appreciated. This work received financial support from Direktoer Jakob Madsen & Hustru Olga Madsens Fund, The Danish Medical Association Research Fund, Bernhard & Marie Kleins Fund, A. P. Moeller & Hustru Chastine Mc-Kinney Moellers Fund, and Institute of Clinical Research, Odense University Hospital.

References

1. **Nerup, J., and A. Lernmark.** 1981. Autoimmunity in insulin-dependent diabetes mellitus. Am. J. Med. **70:**135-141.
2. **The Diabetes Control and Complications Trial Research Group.** 1993. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin dependent diabetes mellitus. N. Engl. J. Med. **329:**977-986.
3. **Anonymous.** 2000. International Islet Transplant Registry, Newsletter No. 8 Draft, on the Occasion of the 7th World Congress of the IPITA, Sidney, Aug 22-25, 1999. ITR,
4. **Shapiro, A. M., J. R. T. Lakey, E. A. Ryan, G. S. Korbutt, E. Toth, N. M. Kneteman, and R. V. Rajotte.** 2000. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N. Engl. J. Med. **343:**230-238.
5. **Fischer, J. S**. 2000. Five little piggies going to market. Will clones be a source of organs for humans? U S News & World Report **128:**51.
6. **van Deijnen, J. H., C. E. Hulstaert, G. H. Wolters, and R. van Schilfgaarde.** 1992. Significance of the peri-insular extracellular matrix for islet isolation from the pancreas of rat, dog, pig, and man. Cell Tissue Res. **267:**139-146.
7. **Ulrichs, K., A. Heiser, V. Eckstein, H. H. Wacker, and A. Thiede.** 1995. Histomorphological characteristics of the porcine pancreas as a basis for the isolation of islets of Langerhans. Xenotransplantation **2:**176-187.
8. **White, S. A., DS. P. Hughes, H. H. Contractor, and N. J. M. London.** 1999. An investigation into the distribution of different collagen types within adult and juvenile porcine pancreata. J. Mol. Med. **77:**79-82.
9. **Ricordi, C., C. Socci, A. M. Davalli, C. Staudacher, P. Baro, A. Vertova, I. Sassi, F. Gavazzi, G. Pozza, and V. Carlo.** 1990. Isolation of the elusive pig islet. Surgery **107:**688-694.
10. **Kirchhof, N., B. J. Hering, V. Geiss, K. Federlin, and R. G. Bretzel.** 1994. Evidence for breed-dependent differences in porcine islets of Langerhans. Transplant. Proc. **26:**616-617.
11. **Heiser, A., K. Ulrichs, and W. Muller Ruchholtz.** 1994. Influence of porcine strain, age, and pH of the isolation medium on porcine pancreatic islet isolation success. Transplant. Proc. **26:**618-620.
12. **Meyer, T., C. Buhler, S. Czub, U. Beutner, C. Otto, A. Thiede, and K. Ulrichs.** 1998. Selection of donor pigs for pancreatic islet transplantation may depend on the expression level of connective tissue proteins in the islet capsule. Transplant. Proc. **30:**2471-2473.
13. **Cavanagh, T. J., J. R. Lakey, M. J. Wright, T. Albertson, K. Wile, and T. J. Fetterhoff.** 1998. Identification of a pig strain with maximal islet mass. Transplant. Proc. **30:**368.
14. **Socci, C., C. Ricordi, A. M. Davalli, C. Staudacher, P. Baro, A. Vertova, M. Freschi, F. Gavazzi, S. Braghi, G. Pozza, and V. Di Carlo.** Selection of donors significantly improves pig islet isolation yield. Horm. Metab. Res. Suppl. **25:**32-34.
15. **Marchetti, P., D. W. Scharp, E. H. Finke, R. Giannarelli, C. J. Swanson, B. J. Olack, P. E. Lacy, and R. Navalesi.** 1994. Factors affecting in vitro and in vivo function of isolated porcine islets. Transplant. Proc. **26:**3419-3420.
16. **Toso, C., D. Brandhorst, J. Oberholzer, F. Triponez, L. Bühler, and F. Morel.** 2000. Isolation of adult porcine islets of Langerhans. Cell Transplant. **9:**297-305.
17. **Miyamoto, M., K. Inoue, Y. Gu, T. Tun, W. Cui, I. Fujiwara, H. Ohyanagi, H. Hayashi, T. Yamazaki, H. Setoyama, Y. Kawakami, J. Ida, M. Kogire, M. Imamura, H. Iwata, and Y. Ikada.** 1998. Improved large-scale isolation of breeder porcine islets: possibility of harvesting from nonheart-beating donor. Cell Transplant. **7:**397-402.
18. **Wright, M. J., T. J. Cavanagh, T. J. Fetterhoff, and K. J. Wile.** 1994. Effect of blood content on porcine pancreatic dissociation and islet yield. Transplant. Proc. **26:**3442
19. **Sun, Y. L., X. Ma, D. Zhou, I. Vacek, and A. M. Sun.** 1993. Porcine pancreatic islets: isolation, microencapsulation, and xenotransplantation. Artif. Organs **17:**727-733.
20. **Cavanagh, T. J. J. R. Lakey, F. Dwulet, M. J. Wright, K. Wile, T. Albertson, and T. Fetterhoff.** 1998. Improved pig islet yield and post-culture recovery using Liberase PI purified enzyme blend. Transplant. Proc. **30:**367.
21. **Brandhorst, H., D. Brandhorst, B. Hering, and R. G. Bretzel.** 1999. Significant progress in porcine islet mass isolation utilizing Liberase HI for enzymatic low-temperature pancreas digestion. Transplantation **68:**355-361.
22. **Linetsky, E., G. Selvaggi, R. Bottino, S. S. Kong, T. Qian, R. Alejandro, and C. Ricordi.** 1995. Comparison of collagenase type P and Liberase during human islet isolation using the automated method. Transplant. Proc. **27:**3264.
23. **Van Suylichem, P. T., G. H. Wolters,and R. van Schilfgaarde.** 1989. The efficacy of density gradients for islet purification. Transplant. Proc. **21:**2624-2625.
24. **Lake, S. P., P. D. Bassett, A. Larkins, J. Revell, K. Walczak, J. Chamberlain, G. M. Rumford, N. J. London, P. S. Veitch, P. R. Bell, and R. F. L. James.** 1989. Large-scale purification of human islets utilizing discontinuous albumin gradient on IBM 2991 cell separator. Diabetes, **38 Suppl 1:**143-145.
25. **White, S. A., A. Swann, D. P. Hughes, H. H. Contractor, and N. J. London.** 1997. Different treatment regimens to reduce microbial contamination of purified porcine islets of Langerhans. Transplantation **64:**770-773.
26. **Urushihara, T., R. Sumimoto, K. Sumimoto, N. V. Jamieson, H. Ito, M. Ikeda, Y. Fukuda, and K. Dohi.** 1992. A comparison of some simplified lactobionate preservation solutions with standard UW solution and Eurocollins solution for pancreas preservation. Transplantation **53:**750-754.
27. **Marchetti, P., R. Giannarelli, A. di Carlo, A. Vassalle, L. Picaro, V. Gattai, L. Pozzolini, A. Masoni, and R. Navalesi.** 1990. Morphometrical and immunocytochemical characterization of the porcine endocrine pancreas. Transplant. Proc. **22:**727-728.
28. **Chadwick, D. R., G. S. Robertson, S. Rose, H. Contractor, R. F. James, P. R. Bell, and N. J. London.** 1993. Storage of porcine pancreatic digest prior to islet purification. The benefits of UW solution and the roles of its individual components. Transplantation **56:**288-293.
29. **Vargas, F., M. Vives Pi, N. Somoza, L. Alcalde, P. Armengol, M. Marti, L. Serradell, M. Costa, J. Fernandez Llamazares, A. Sanmarti, and R. Pujol Borrell.** 1996. Advantages of using a cell separator and metrizamide gradients for human islet purification. Transplantation **61:**1562-1566.
30. **Bretzel, R. G., R. Alejandro, B. J. Hering, P. T. Van Suylichem, and C. Ricordi.** 1994. Clinical islet transplantation: guidelines for islet quality control. Transplant. Proc. **26:**388-392.
31. **Kofoed, H.** 1992. Secretin and the endocrine pancreas. Acta Endocrinol. **126:**1-42.
32. **Csernus, V.J., T. Hammer, D. Peschke, and E. Peschke.** 1998. Dynamic insulin secretion from perfused rat pancreatic islets. Cell Mol. Life Sci. **54:**733-743.
33. **Hemmilä, I., S. Dakubu, V. Makkala, H. Siitari, and T. Lövgren.** 1984. Europium as a label in time-resolved immunofluorometric assays. Anal. Biochem. **137:**335-343.
34. **Calafiore, R., F. Calcinaro, G. Basta, M. Pietropaolo, A. Falorni, M. Piermattei, and P. Brunetti.** 1990. A method for the massive separation of highly purified, adult porcine islets of Langerhans. Metabolism **39:**175-181.

35. **Marchetti, P., R. Giannarelli, G. Villani, M. Andreozzi, L. Cruschelli, S. Cosimi, P. Viacava, A. di Carlo, R. Navalesi, and G. Bevilacqua.** 1994. Collagenase distension, two-step sequential filtration, and histopaque gradient purification for consistent isolation of pure pancreatic islets from the market-age (6-month-old) pig. Transplantation **57:**1532-1535.
36. **Prevost, P., E. Rolland, C. Veriot, C. Collier, and E. Muscat.** 1995. Large-scale isolation of porcine pancreatic islets: significant improvement of the process. Transplant. Proc. **27:**3396-3398.
37. **Warnock G. L., D. Katayal, J. R. T. Lakey and R. V. Rajotte.** 1995. Studies of the isolation, viability, and preservation of purified islets after surgical pancreatectomy in large pigs. Xenotransplantation **2:**161-164.
38. **Abalovich, A., I. Mignone, C. Susemihl, C. Ricci, S. Migliore, R. Rodriguez, C. Brignone, and J. Brignone.** 1995. Preparation of pig pure islets without using density gradients. Transplant. Proc. **27:**3353.
39. **Gray, D. W.** 1999. Porcine islet isolation: a real advance? [comment]. Transplantation **68:**325-326.
40. **Heald, K. A., T. Jay, and R. Downing.** (1993). Cold ischaemia reduces porcine islet yields. Horm. Metab. Res. **25:**57(abstract).
41. **Klock, G., M. B. Kowalski, B. J. Hering, M. E. Eiden, A. Weidemann, S. Langer, U. Zimmermann, K. Federlin, and R. G. Bretzel.** 1996. Fractions from commercial collagenase preparations: use in enzymic isolation of the islets of Langerhans from porcine pancreas. Cell Transplant. **5:**543-551.
42. **Ricordi, C., P. E. Lacy, and D. W. Scharp.** 1989. Automated islet isolation from human pancreas. Diabetes **38** (Suppl. 1)**:**140-142.
43. **Downing, R., T. R. Jay, D. T. Topham, J. Webberley, and K. A. Heald.** 1995. Isolation and function of islets from 6-week-old gnotobiotic pigs. Transplant. Proc. **27:**3331-3332.
44. **Heald, K. A., T. R. Jay, and R. Downing.** 1997. Insulin secretory studies on isolated pig islets. Transplant. Proc. **29:**2021-2022.
45. **Hesse, U. J., G. P. Meyer, J. Weyer, J. Danis, and H. Pichlmaier.** 1994. Islet isolation and autotransplantation in pigs. Zentralbl. Chir. **119:**653-660.
46. **Brandhorst, D., H. Brandhorst, B. J. Hering, and R. G. Bretzel.** 1999. Long-term survival, morphology and in vitro function of isolated pig islets under different culture conditions. Transplantation **67:**1533-1541.
47. **Holmes, M. A., H. A. Clayton, D. R. Chadwick, R. P. Bell, N. J. London, and R. F. James.** 1995. Functional studies of rat, porcine, and human pancreatic islets cultured in ten commercially available media. Transplantation **60:**854-860.
48. **Marchetti, P., D. W. Scharp, K. Pfiffner, C. J. Swanson, E. H. Finke, B. J. Olack, A. Gerasimidi Vazeou, M. McLear, and P. E. Lacy.** 1994. Cryogenic storage of isolated, purified porcine pancreatic islets. Transplantation **57:**340-346.
49. **Davalli, A. M., F. Bertuzzi, C. Socci, L. Scaglia, F. Gavazzi, M. Freschi, V. DiCarlo, A. E. Pontiroli, and G. Pozza.** 1993. Paradoxical release of insulin by adult pig islets in vitro. Transplantation **56:**148-154.
50. **Bertuzzi, F., C. Berra, C. Socci, A. M. Davalli, G. Calori, M. Freschi, L. Piemonti, P. De Nittis, G. Pozza, and A. E. Pontiroli.** 1995. Glucagon improves insulin secretion from pig islets in vitro. J. Endocrinol. **147:**87-93.
51. **Gouin, E., A. S. Rivereau, S. Darquy, E. Larher, S. You, A. Jestin, G. Reach, and P. Sai.** 1998. Perifusion analysis of insulin secretion from specific pathogen-free large-white pig islets shows satisfactory functional characteristics for xenografts in humans. Diabetes Metab. **24:**208-214.
52. **Thams, P., and K. Capito.** 1999. L-Arginine stimulation of glucose-induced insulin secretion through membrane depolarization and independent of nitric oxide. Eur. J. Endocrinol. **140:**87-93.
53. **Ricordi, C., D. W. Gray, B. J. Hering, D. B. Kaufman, G. L. Warnock, N. M. Kneteman, S. P. Lake, N. J. London, C. Socci, R. Alejandro, Y. Zeng, D. W. Scharp, G. Viviani, L. Falqui, A. Tzakis, R. G. Bretzel, K. Federlin, G. Pozza, R. F. L. James, R. V. Rajotte, V. Di Carlo, P. J. Morris, D. E. R. Sutherland, T. E. Starzl, D. H. Mintz and P. E. Lazy.** 1990. Islet isolation assessment in man and large animals. Acta Diabetol. Lat. **27:**185-195.
54. **Davalli, A. M., Y. Ogawa, L. Scaglia, Y. J. Wu, J. Hollister, S. Bonner Weir, and G. C. Weir.** 1995. Function, mass, and replication of porcine and rat islets transplanted into diabetic nude mice. Diabetes **44:**104-111.
55. **Maki, T., J. J. O'Neil, J. Porter, C. J. Mullon, B. A. Solomon, and A. P. Monaco.** 1996. Porcine islets for xenotransplantation. Transplantation **62:**136-138.
56. **Vajkoczy, P., A. M. Olofsson, H. A. Lehr, R. Leiderer, F. Hammersen, K. E. Arfors, and M. D. Menger.** 1995. Histogenesis and ultrastructure of pancreatic islet graft microvasculature. Evidence for graft revascularization by endothelial cells of host origin. Am. J. Pathol. **146:**1397-1405.
57. **Menger, M. D., S. Jaeger, P. Walter, G. Feifel, F. Hammersen, and K. Messmer.** 1989. Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans. Diabetes, **38** (Suppl. 1)**:**199-201.
58. **Beger, C., V. Cirulli, P. Vajkoczy, P. A. Halban, and M. D. Menger.** 1998. Vascularization of purified pancreatic islet-like cell aggregates (pseudoislets) after syngeneic transplantation. Diabetes **47:**559-565.