Pancreatic islet transplantation: studies on the technique and efficacy of islet isolation and transplantation

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3 Assessment of islet isolation efficacy in dogs*

Introduction

The recent progress in islet isolation techniques has accentuated the need for further refinement of the methods for assessment of isolation efficacy. The outcome of islet isolation is considered uncertain because of the large variability of insulin recovery, size and yield of isolated islets [10,12,19,33]. In the past, the efficacy of isolation techniques has been estimated indirectly, by assessment of insulin recovery from the donor pancreas [13,14,17,21,36]. However, because islet and insulin recovery are generally expected to be affected differently by the many variables during isolation, insulin extraction has been largely superseded by the current method of sizing islets to assess the total volume of harvested islets. Morphometric assessment of islet yield has facilitated the comparison of results from different laboratories [12,20,26] and definition of the critical islet mass for successful transplantation [8,15,42]. As yet, no attempts have been made to extend the use of morphometry for assessment of isolation efficacy by comparison of islet yield with the native islet population of the pancreas — and, consequently, no comparison of islet and insulin recovery has been made either. We therefore addressed the assessment of islet isolation efficacy by both morphometry and extraction of insulin and amylase, and substantiated the viability of isolated islets by in vitro perifusion.

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* Michael P.M. van der Burg, Onno R. Guicherit, Marijke Frölich, Johannes P. Scherft, Frans A. Prins, Jan Anthonie Bruijn, and Hein G. Gooszen. *Cell Transplant* 3: 91–101, 1994

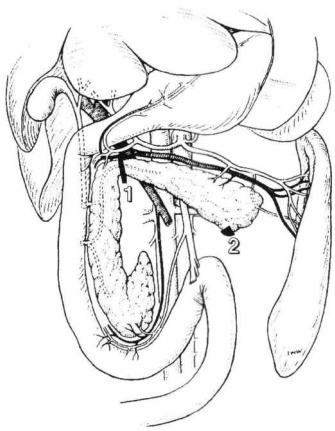

Fig. III.1

Schematical representation of the beagle pancreas. In control dogs the insulin distribution in the splenic pancreas was studied from biopsies at sites 1 and 2. In experimental dogs the pancreas was transected at site 1, and a biopsy specimen was taken from the cut end for assessment.

Materials and methods

Design of the study

We report the results from 31 consecutive islet isolations from the splenic segment of the dog pancreas. First, the insulin distribution in the splenic pancreas was studied in a separate group (n=9) from biopsies at site 1 (corresponding to plane of cleavage of the pancreas in the experimental group) and site 2 (Fig. III.1). Insulin values from site 2 amounted to 107±9% (NS) of values obtained from site 1. Therefore, specimens from the cut end of the splenic segment were taken to represent this segment in our experimental group. Islet isolation was performed by intraductal stationary collagenase digestion, using the same batch of collagenase throughout the study. Tissue was manually dispersed in cold (4°C) University of Wisconsin solution (UWS) and the resulting digest purified by density gradient centrifugation. We used three different density gradients for non-related studies, and pooled the results in this study because a similar purity, and similar distributions of islets and insulin were observed in the purified (Pure) and nonpurified (Rest) fractions of the gradients (Fig. III.2).

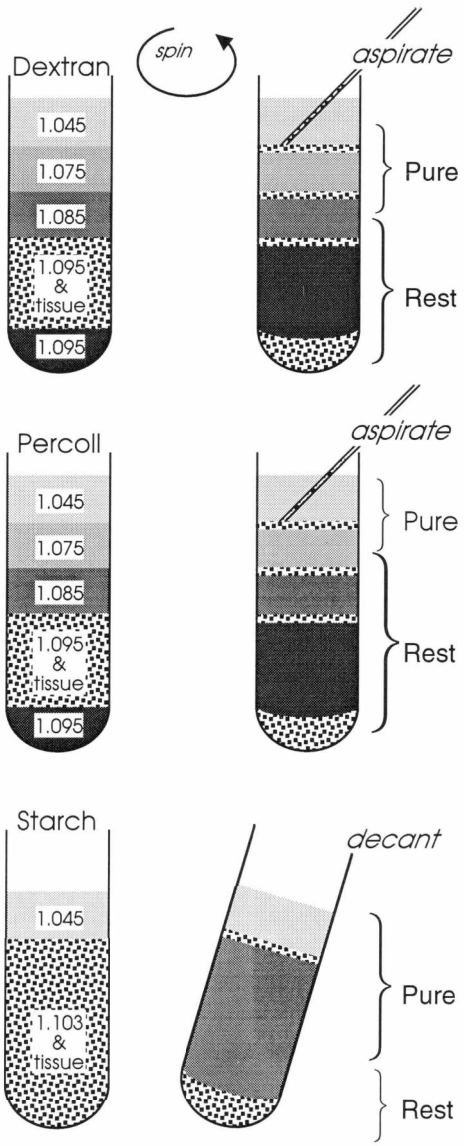

Fig. III.2

Schematical representation of separation of >90% purified islets (Pure) and nonpurified tissue (Rest) using either discontinuous isodense gradients of dextran (n = 15) and Percoll (n = 10), or neutral density centrifugation in a starch solution (n = 6).

Samples from the pancreas, the digest suspension, and gradient fractions, were compared by morphometry of islet volume and the islet size distribution, by insulin and amylase extraction, and by microscopy for morphology and to assess ß-cell granulation as a parameter for the insulin content. Viability of isolated islets was assessed by glucose stimulation during in vitro perifusion.

Media for islet isolation

The collagenase solution (pH 7.5) contained 5% (w/v) hydroxyethyl starch (DCC-607 Pentastarch solution, Du Pont Critical Care, Waukegan, IL, USA), 20 mM Hepes, 4.5 mM sodium bicarbonate, 90% (w/v) of the regular Hanks' balanced salts (Flow Laboratories, Irvine Ayrshire, Scotland) and 1633 U/ml collagenase (collagenase type XI, Sigma, St. Louis, MO, USA). The UWS (ViaSpan™, Du Pont Pharmaceuticals, Wilmington, Delaware, USA) was used without the regular additives insulin and dexamethasone, and supplemented with 0.4% (w/v) bovine serum albumin (fraction-V, 35%-solution in PBS, Sigma). No albumin was added to the UWS used for preparation of the gradients and washing after density separation. Dextran (D3759, average mol wt 71 kD, Sigma; or dextran T70, Pharmacia, Uppsala, Sweden) density solution (density 1.095 g/ml) was prepared in Hanks' balanced salt solution (HBSS), supplemented with 20 mM Hepes (pH 7.4), and autoclaved for 5 min at 120°C. Percoll (Pharmacia, Uppsala, Sweden) density solution (density 1.095 g/ml) was prepared in a basal UWS containing regular amounts of potassium lactobionate, KH_2PO_4, $MgSO_4$, D-raffinose, and 4% (w/v) Pentastarch. Final pH was adjusted to 7.4 with NaOH, and dilutions for other densities were made with UWS. Starch (Pentastarch Powder, Du Pont Pharmaceuticals, Wilmington, Delaware, USA) density solution (density 1.103 g/ml) was prepared in UWS, and filter-sterilized. RPMI 1640 medium was supplemented with 4.2 mM sodium bicarbonate, 20 mM Hepes, 2 mM L-glutamine, and 10% (v/v) heat-inactivated fetal calf serum (Flow Laboratories).

Islet isolation

After a 24 h fast, female, outbred beagles (Harlan CPB, Zeist, The Netherlands) weighing 12.4±0.5 kg (range 8–18 kg) were anesthetised as described previously [9]. The splenic segment of the pancreas was mobilized and the pancreas was divided, where it overlies the portal vein. A specimen was taken from the cut end for morphometry and extractions (Fig. III.1). The duct was cannulated with a 20-gauge stub adapter, and the gland was removed, weighed, and within 90 s from the onset of ischemia, perfused (flow 1 ml/min per g pancreas) for 5–10

min with 150 ml of an ice cold, recirculating, collagenase solution, resulting in a fourfold increase of the volume of the gland. The gland was covered with the remaining collagenase solution, transported on ice to the islet isolation facility, and within 40 min incubated at 38°C for 20 min in a waterbath. The collagenase solution was decanted, pancreatic weight was corrected for the weight of the stub adapter and adhering nondigested tissue of the cut end of the gland (8 ± 1%), and the tissue was dispersed in ice cold UWS by gentle aspiration and flush through a blunt, 14-gauge, needle, and filtration through a 400 μm steel mesh. Trapped tissue was syringed and sieved again for 1–2 times and the final trapped tissue — which largely consisted of ductal and vascular fragments — was blotted, weighed, and discarded. The ~1000 ml suspension was centrifuged for 2 min at 4°C and 200 *g* in a Beckman J-6M/E centrifuge (JS3.0 rotor; Beckman Instruments, Palo Alto, CA, USA) in 50-ml screw cap conical tubes (Falcon®, Becton Dickinson Labware, Lincoln Park, New Jersey). Sediments were pooled into one tube and spun for measurement of the digest packed-tissue-volume (PTV). After resuspension, ~1 ml samples for assessment were transferred to microfuge tubes on ice, and the digest aliquoted in tubes (PTV < 2 ml per tube) for density gradient purification. After centrifugation and decantation the pelleted digest was resuspended by gentle passage through a 10-ml pipet in a 12 ml solution (density 1.095 g/ml) of dextran (n = 15) or Percoll (n = 10), or a 1.103 g/ml density solution of starch (n = 6). The starch solution was topped with UWS; and the dextran or Percoll solutions were underlaid with another 4 ml of these density solutions, and overlaid with 6 ml each of these solutions with densities of 1.085, 1.075 and 1.045 g/ml resp. (Fig. III.2). Gradients were centrifuged at 4°C, 40 *g* for 5 min, and 500 *g* for 12 min, without braking. Purified islets were collected from the two uppermost interfaces of the dextran gradient, the top interface of the Percoll gradient, and collected by decantation of the starch density solution. The purified islets (pure fraction) and the remainder of the gradients (rest fraction) were washed thrice in UWS for resp. 4, 2, and 2 min at 200 *g* . After taking aliquots for assessment, the purified islets were washed in RPMI, aliquoted (< 2 μl PTV in 5 ml RPMI) to bacteriological petri plates (10 cm in diameter), and cultured at 36 °C in a humidified ambient atmosphere.

Perifusion studies

After overnight culture, aliquots containing 1 μl islets (range 0.3–3.3) were transferred to six 0.5-ml microchambers to conduct three duplicate perifusion tests in parallel in the Acusyst-S™ perifusion system (Endotronics, Coon

Rapids, MN, USA). Media were drawn via 3-way disposable stopcocks at 0.25 ml/min, heated to 36°C and gassed with medical grade air. Each experiment consisted of an initial 90-min equilibration period with 2.5 mM glucose, followed by a 40-min period of 2.5, 7.5, or 10 mM glucose perifusion. From 30 min before stimulation fractions were collected at intervals as indicated in the figures, and stored at -20°C pending insulin assay. The basic perifusion fluid was Krebs-Ringer-Hepes containing 4.2 mM $NaHCO_3$, 10 mM Hepes, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM KCl, 1.2 mM NaH_2PO_4, 118 mM NaCl (at 0 mM glucose), and 0.2% (w/v) bovine albumin fraction V (cell culture tested; Sigma). The sodium chloride content was adjusted to the amount of glucose added to maintain the osmolarity constant. Insulin secretion data from duplicate chambers were averaged and expressed as a percentage of the averaged three values for basal secretion from -15 to 0 min.

Morphometry

Pancreatic biopsy specimens were fixed in Bouin's solution and embedded in paraplast. Ten serial sections (5 µm thick) taken at 150 µm intervals were stained with hematoxylin-eosin for measurement of the fractional islet volume with the grid method for point-counting, assuming uniform shrinkage of islet and nonislet tissue [43]. At least 40,000 points (mean: 116,250 ± 9917) per specimen (relative standard error < 5%) were examined, using a 400-point lattice grid at x200 final magnification on systematically chosen nonoverlapping fields that covered the tissue section, with exclusion of the capsular and interlobular area. Islet volume was expressed per gram pancreas (assuming a density of 1.00 g/ml). Specimens (n = 10) containing at least 200 islet profiles in sections taken at intervals of 450 µm were selected for islet sizing to derive the islet size distribution. The profile areas were measured by planimetry using the grid at x200 final magnification. Measurements were corrected for shrinkage during histological processing, using a linear shrinkage factor of 0.74 (mean value of 5 experiments). Assuming islets to be spheres, the diameter of the equivalent circular area was calculated. These profile diameters were grouped into 25 µm classes and analysed by the Saltykov diameter approach of 1958 [43] as modified by Cruz-Orive [6] to obtain the number of islets per cm3 pancreas for each size class. For comparison with isolated islets, the first class (diameters < 37.5 µm) was excluded from calculation of the total number of islets and a percentage for each size class (numerical size distribution). The maximum and mean size (number-average diameter) of islets were used as parameters for the numerical size distribution. The mean size was

calculated by multiplying the mean diameter of each class by the number of islets for that class, summing the answers, and dividing by the total number of islets. Using the mean diameter of each class the volume size distribution was derived, and likewise the volume-average diameter calculated.

Duplicate 50-µl aliquots of the fresh islet suspensions were stained with 20 µl dithizone (0.65 mM diphenylthiocarbazone and 4.5% ethanol in PBS) on a microscopic slide. The mean diameters (≥ 40 µm) of all dithizone-stained islets, fully cleaved from acinar tissue, were recorded in increments of 10 µm using an ocular micrometer by transmission microscopy at x100 magnification. Islets were categorized into 25 µm diameter classes (> 37.5 µm) and considered spheres for determination of volume. The morphometric parameters investigated were similar as described for pancreas specimens: the maximum diameter, number-average diameter, total volume of isolated islets per gram processed pancreatic tissue (µl/g), and the volume-average diameter. The equivalent number of islets (IEq) was obtained by conversion of the total islet volume, assuming a standard islet diameter of 150 µm [26]. The total volume of aliquots examined for islet sizing averaged 0.11 ± 0.01% of an islet suspension, and contained 196 ± 25 islets with a total 74 ± 11 nl islet volume. The coefficient of variation for duplicate measurements of islet volume was 17% (n = 72).

Purity was expressed as the fractional islet volume of islet and acinar tissue — and was estimated from the morphometrically measured islet volume and the amount of amylase, as a measure of the acinar volume. The acinar volume corresponding to amylase recovery after purification, was extrapolated from the ratio of amylase and fractional acinar volume of the packed-tissue in corresponding digest preparations, assuming absence of fibrous tissue.

Reflection Contrast Microscopy

Aliquots of islet suspensions obtained during isolation were centrifuged (50 g, 15 s, 20°C), the supernatant was aspirated and the sediment mixed with 30 µl cock plasma and 5 µl chicken embryonic extract to initiate clotting, centrifuged (50 g, 15 s, 20°C), incubated for 3–5 min at room temperature and fixed. Specimens for reflection contrast microscopy were processed as described in detail previously [5]. Briefly the pancreatic specimens or isolated islets entrapped in plasma clots, were fixed in 4% freshly prepared paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After dehydration in dimethylformamide and Lowicryl-embedding at room temperature ultrathin (50–70 nm) sections were placed on aminosilane coated slides. After washing in 0.5 M NH_4Cl in PBS and preincubation with 1% (w/v) bovine serum albumin

in PBS for 15 min at 20°C the sections were first incubated 16 h at 4°C with rabbit-α-human insulin IgG (1:20) and next, after washing, incubated 2 h at 20°C with 15-nm gold-conjugated goat-α-rabbit IgG (1:40). Specimens were not counterstained and examined with an incident light microscope with a 75 W Xenon lamp (Leitz Orthoplan; Leitz, Wetzlar, Germany) equipped with special reflection contrast devices.

Insulin and amylase assay.

Pancreatic samples were thawed, divided into pieces of ~40 mg, weighted and homogenized (at setting 8; Polytron PT10, Lucerne, Switzerland) for 20 s at 4°C in 1 ml acid-alcohol (0.15 M HCl, 77% ethanol) for insulin extraction, or 4 ml HBSS (20 mM Hepes) for amylase extraction. Samples obtained during islet isolation were thawed, a 100 µl aliquot plus 400 µl acid-alcohol for insulin, and 300 µl aliquots for amylase, were sonicated in microfuge tubes on ice for 20 s (Sonicator™W375, amplitude set at microtip limit, Ultrasonics Inc., Plainview, NY). Homogenates were extracted overnight at 4°C, centrifuged, and the supernatant either stored at -20°C pending radioimmunoassay, or assayed immediately for amylase by colorimetric assay (Phadebas® α-Amylase Test, Pharmacia, Uppsala, Sweden). Acid-alcoholic extracts were assayed after ≥100-fold dilution with assaybuffer. Insulin was measured by a radioimmunoassay previously reported [9] using dog insulin as standard.

Statistical Analysis.

Results are expressed as mean ± SE. Differences between means were analysed by single factor analysis of variance with repeated measures and multiple comparisons were performed by Scheffé's test. Differences were considered not significant (NS) at $p > .05$. With linear regression and correlation analysis differences were considered NS at $p > .01$.

Results

Results are reported in Table III.1. The weight of pancreatic tissue processed for islet isolation amounted to 9.2 ± 0.4 g. Digest packed-tissue-volume (PTV) indicated a mean 79% recovery of processed tissue. Taking into consideration wasted tissue trapped with sieving, which averaged 0.6 ± 0.1 g, PTV corresponded to 85 ± 1% of the expected tissue volume. Both amylase and insulin recovery reflected the loss of tissue during isolation. After digestion 95% of pancreatic amylase and 89% of pancreatic insulin were recovered. In contrast to these biochemical parameters, the morphometrically assessed volume of isolated islets in the digest (7.6 ± 0.7 µl/g) corresponded to a 49% recovery of pancreatic islets. It should be noted that islets (partially) entrapped in acinar tissue were not included in our counts. Subjectively, the volume of entrapped islets was estimated to amount to 20% — at most — of the total

Table III.1

Recovery of pancreatic tissue, amylase, insulin, and islets after collagenase digestion (Digest) and density gradient centrifugation in the purified fraction (Pure) and rest of the gradient (Rest)

	Pancreas	Digest		Pure		Pure & Rest	
Tissue vol (ml)	9.2 ± 0.4	7.2 ± 0.3*	(79 ± 1)	<0.1*		5.2 ± 0.5†‡	(78 ± 9)
Amylase (IU/g)	7097 ± 690	6706 ± 771	(95 ± 5)	1.3 ± 0.3*	(.02 ± .01)	3939 ± 396*	(64 ± 6)
Insulin (nmol/g)	21.6 ± 1.5	18.7 ± 1.3†	(89 ± 7)	6.5 ± 0.7*	(36 ± 3)	15.3 ± 1.2†	(83 ± 5)
Islet volume (µl/g)	15.7 ± 0.9	7.6 ± 0.7*	(49 ± 4)	3.9 ± 0.5*	(53 ± 3)	4.6 ± 0.5*	(64 ± 3)
Islets (IEq.10^3/g)§	8.9 ± 0.5	4.3 ± 0.4*		2.2 ± 0.3*		2.6 ± 0.3*	
Islet size (µm)‖						ND	
largest diameter	192 ± 13	189 ± 12		183 ± 13			
number-average	75 ± 2	70 ± 1		73 ± 2			
volume-average	106 ± 5	119 ± 6		120 ± 7			
Insulin/Islet (nmol/µl)	1.5 ± 0.1	2.9 ± 0.2*	(190 ± 13)	1.8 ± 0.2*	(68 ± 5)	ND	
Purity: vol (%)	1.57 ± 0.09	ND		94 ± 1		ND	

Values are means ± SE (n = 31) with % recovery in parenthesis. Amylase, insulin, islet volume and number (rows 2–5) are given per gramme pancreas. For Digest values significance of difference and % recovery are compared to Pancreas values. Similarly Pure and Pure & Rest values were compared with Digest values. ND, Not determined. * $p < .001$, † $p < .05$.
‡ Data from 8 experiments after dextran purification; analysis by paired comparison with Digest values. § The number of islet equivalents (IEq) is the number of islets with diameter 150 µm, corresponding to the islet volume obtained. ‖ Islet size parameters analysed within subject in 10 experiments

volume of dithizone stained tissue. Islet yield after digestion (range 1.9–15.9 μl/g) nevertheless, did correlate well ($r = .63$, $p < .0001$) with insulin yield (range 9.6–39.2 nmol/g). Similarly, a highly significant correlation ($r = .74$, $p < .0001$) was demonstrated between the islet content (range 8.4–27.3 μl/g) and insulin content (range 11.5–41.5 nmol/g) of pancreatic specimens. The islet content of pancreases further correlated well with islet yield ($r = .69$, $p < .0001$) and insulin yield ($r = .57$, $p < .001$) after digestion. Similar findings were obtained for pancreatic insulin: the insulin content of the pancreas correlated well with both islet yield ($r = .75$, $p < .0001$) and insulin yield ($r = .71$, $p < .0001$) in the digest suspensions. Recovery — expressed as a percentage of digest values — in the pure fraction *vs* the combined pure & rest gradient fractions (Table III.1) amounted to: ~0 *vs* 80% for tissue volume, 0 *vs* 64% for amylase, 36 *vs* 83% for insulin, and 53 *vs* 64% for islet volume. Thus, most of the islets were harvested from the pure fraction of the gradients, while half of the total amount of recovered insulin was located in the rest fraction of the gradients. The islet content of the pure fraction correlated ($r = .59$; $p < .001$) with the insulin content. Correlations of the islet and insulin content in the digest and pure fractions of the gradients are illustrated in Fig. III.3. The islet content of the digest suspension correlated well ($p < .0001$) with the islet ($r = .71$) and insulin content ($r = .65$) of the pure fraction (Figs. III.3A,B), as well as with the total volume of islets in the combined pure and rest fractions of the gradients ($r = .58$; $p < .001$). In contrast, the insulin content of digest suspensions did neither correlate with islet ($r = .34$) or insulin recovery ($r = .40$) in the purified suspensions (Figs. III.3C,D), nor with total islet recovery in the combined pure and rest gradient fractions ($r = .37$), but did correlate well with insulin recovery in the combined pure and rest gradient fractions ($r = .63$; $p < .0001$).

Comparison of parameters for the islet size distributions of the pancreas, digest and purified suspensions demonstrated no differences for either the largest and average islet diameter (number-average diameter), or the volume-average diameter (Table III.1). Size distributions of islets in pancreas, digest and purified preparations are shown in Fig. III.4. Due to the restricted number of islet profiles available for sizing in sections of pancreatic specimens, these data were obtained in 10 experiments only. Size analysis of isolated islets in all experiments demonstrated significant correlations between the islet content of the pancreas (μl/g) and the maximum ($r = .53$) or volume-average diameter ($r = .46$) of islets in the digest suspensions ($p < .01$). Significant correlations were also found between yield (μl/g) and the maximum ($r = .75$) or volume-average diameter ($r = .78$) of islets in the digest suspensions ($p < .0001$). The maximum diameter of these islets further correlated with body wt of the animals ($r = .44$, p

= .01).

The ratio of insulin and islet volume per gram (processed) pancreas was used to estimate the insulin content of islets. Similar values for purified islets (1.8 ± 0.2) and the native endocrine pancreas (1.5 ± 0.1; NS) demonstrated no loss of insulin from islets during isolation. Comparison by reflection contrast microscopy (n = 3) of ultrathin sections of the pancreas and purified-islet sediments stained for insulin with the immuno-gold technique (Fig. III.5) demonstrated a similar degree of ß-cell granulation.

Viability of overnight cultured islets was demonstrated by glucose-stimulation during perifusion. Basal insulin secretion prior to stimulation averaged 0.08 ± 0.01% of the insulin content of islets per hour, corresponding to 26 ± 5 fmol/min per µl islet volume or 0.0054 ± 0.0005 µU/min per IEq. Glucose

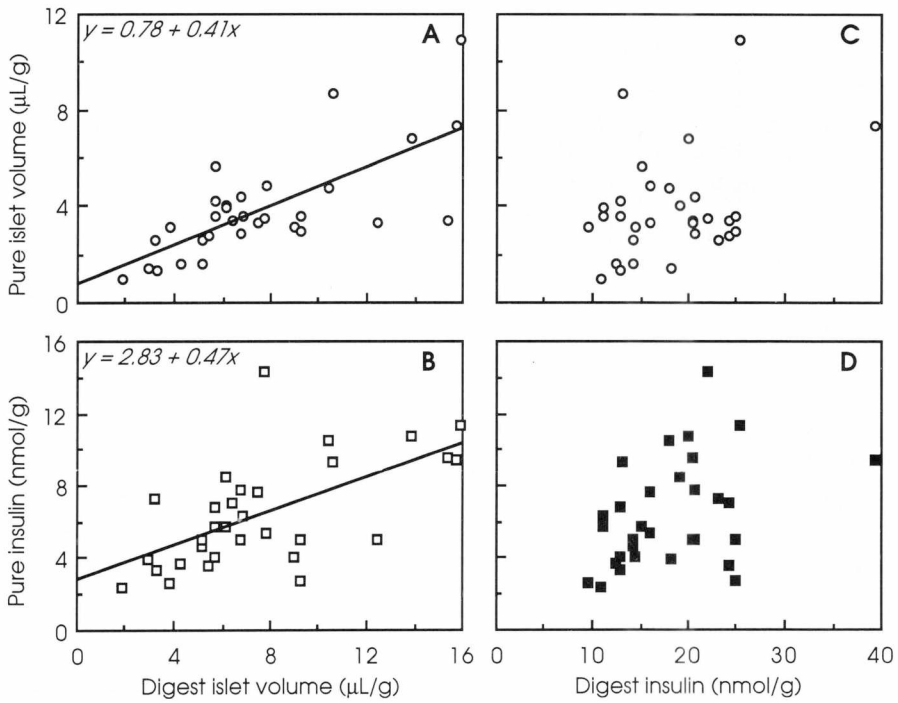

Fig. III.3

*Assessment of islet and insulin recovery in pure fractions after density gradient centrifugation. The morphometrical assessed islet content of digest suspensions correlated significantly ($p < .0001$) with the islet content (**A**) and insulin content (**B**) of purified suspensions; $r = 0.71$ and 0.65 respectively. No correlation was evident between the insulin content of digest tissue and the islet (**C**) or insulin (**D**) content of purified suspensions.*

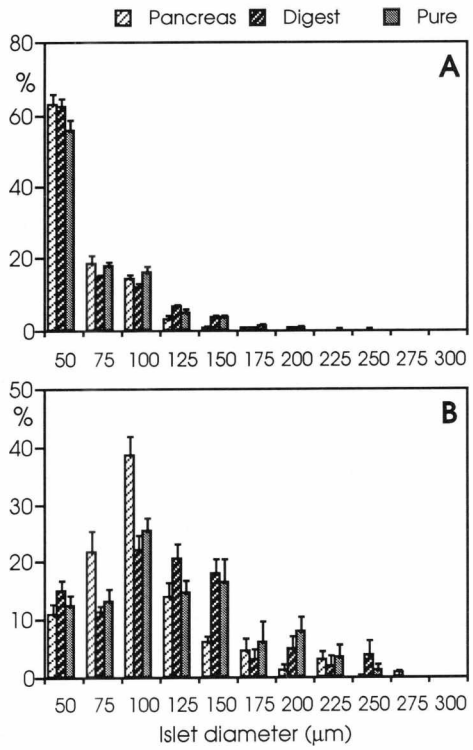

Fig. III.4

*Comparison of the size distributions of islets in the splenic pancreas, after isolation in the digest and in the pure fraction after density gradient centrifugation. No significant differences were demonstrated for the maximum diameter of islets, or the mean size of islets in the numerical (**A**) and volume (**B**) size distributions. Results are expressed as mean ± SE (n = 10).*

stimulation resulted in a biphasic insulin response from 2 min to ~5x basal secretion at 7.5 mM and ~10x basal secretion at 10 mM glucose (Fig. III.6; $n = 6$; $p < .001$).

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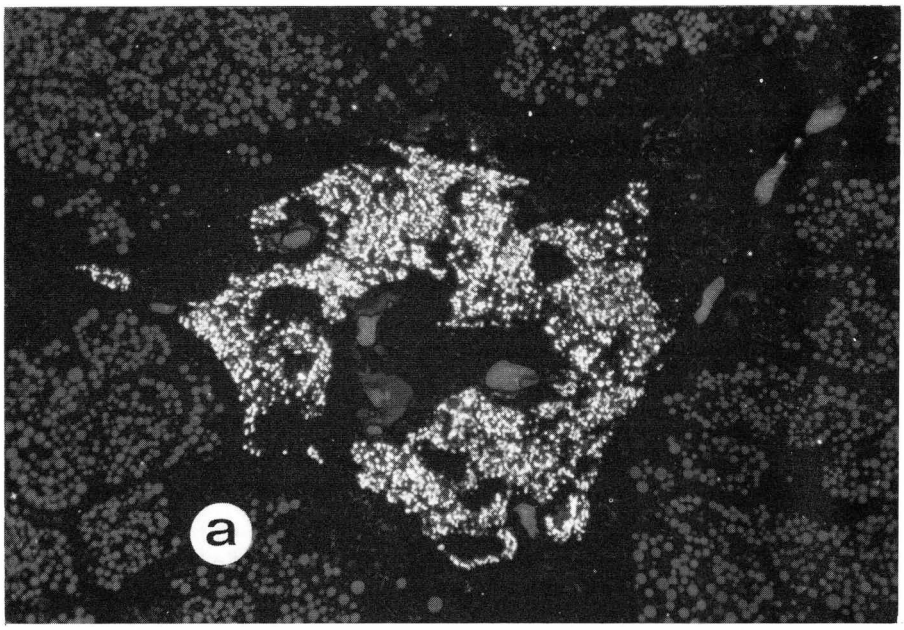
Fig. III.5

*Reflection contrast micrographs of sections of the pancreas (**A**) and density gradient purified islets isolated from this pancreas (**B**), stained by the immuno-gold method for insulin, demonstrating similar ß-cell granulation. Not counterstained. x 630.*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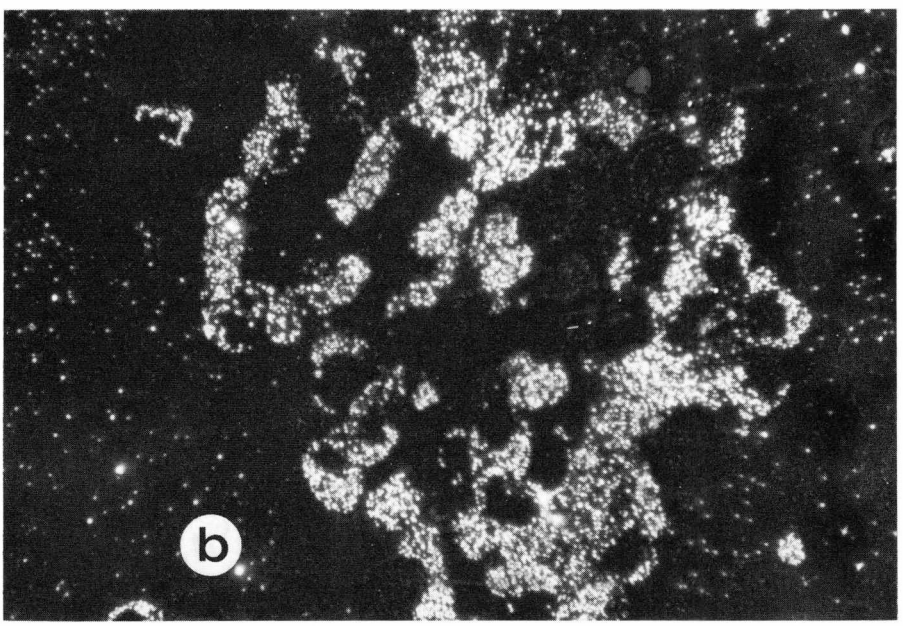
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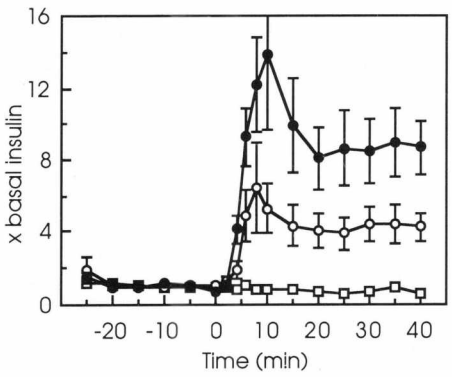

Fig. III.6

Dynamics of insulin release during in vitro glucose stimulation of overnight cultured islets. Aliquots of the islet preparation were perifused in parallel, initially with 2.5 mM glucose and from 0 to 40 min with 2.5 (squares), 7.5 (open circles), or 10 (closed circles) mM glucose. A biphasic insulin response was observed from 2 min to ~5x basal secretion at 7.5 mM, and ~10x basal secretion at 10 mM glucose ($p < .001$, $n = 6$).

Discussion

The variability of islet isolation outcome has been difficult to analyse, due to the intertwined effects of numerous variables, such as predonation events, pancreas characteristics, organ preservation conditions, and isolation methods. Knowledge of the variability attributable to intrinsic factors like the islet and insulin content of the individual pancreas is essential for analysis of the relative importance of extrinsic factors. We therefore compared the isolated and native islet populations, and addressed the efficacy of isolation by both morphometry and extraction — using a simple and gentle technique of islet isolation in dogs, to exclude extrinsic factors as much as possible. Our major finding was, that the variability of islet yield and size may be attributed to a large extent to the variability of the native islet population. Isolation efficacy was best documented by morphometry, because insulin recovery did not discriminate between isolated islets and islets entrapped in acinar tissue. Assessment by both morphometry and insulin extraction, however, demonstrated that we subjectively had underestimated the proportion of entrapped islets during islet sizing and further documented islet integrity by demonstrating preservation of the insulin content of isolated islets.

Only fully cleaved islets were considered for assessment of islet recovery. Although dithizone staining did allow the identification of islets entrapped in acinar fragments, sizing of these islets is difficult at best — and was not attempted, because entrapped islets settled in the acinar fraction after density separation, and moreover, were subjectively estimated to amount to 20% — at most — of the total volume of islet tissue. Correlation analysis demonstrated that ~50% (r^2) of the variance of both islet and insulin yield after collagenase

digestion may be attributed to interindividual differences in the islet and insulin content of the splenic pancreas. Thus, the ~90% recovery of pancreatic insulin as opposed to a ~50% recovery of pancreatic islets after collagenase digestion would indicate a — in contrast to our subjective findings — rather large proportion of entrapped islets. The uneven distribution of islets and insulin in the pure and rest fractions of density gradients supported this explanation. While most of the total volume of free islets recovered in both pure and rest fractions, was found in the pure fraction, roughly half of the total amount of recovered insulin was located in the rest fraction — representing mainly entrapped islets, because entrapped islets were clearly absent in the pure fractions. Similarly, the correlation of the islet and insulin content in the purified fraction of the gradients with the islet content but not with the insulin content of digest suspensions, may be attributed to a variable proportion of entrapped islets in the digest. Theoretically degranulation of ß-cells during islet isolation could also have contributed to the poor correlation of the insulin content of digest and pure islet suspensions. No evidence for degranulation was found, however, neither by comparison of the insulin content of islets in the pancreas and after purification, nor by comparison of ß-cell granulation using reflection contrast microscopy.

Islet and insulin recovery are generally expected to be affected differently by the many variables before and during islet isolation. Indeed, insulin recovery did not reflect the volume of isolated islets in our study — due to the presence of entrapped islets during isolation. However the high recovery of insulin, and the intact insulin content of purified islets clearly indicated that insulin did accurately reflect the volume of islet tissue during isolation. Several factors may have been conducive to these results. By using the female laboratory beagle we excluded variation due to sex, breed, and diet — which may affect the islet and insulin content of the pancreas [11,24] as well as the efficacy of collagenase digestion [18]. We further excluded variation by minimizing warm and cold ischemia of the pancreas, which have been shown to reduce the insulin content, size, and yield of islets [1,19,27,45], and by using a simple and gentle isolation technique and the same batch of collagenase throughout the study. A similar 80–95% recovery of both pancreatic tissue volume, insulin and amylase after digestion in our study compares favourably with previous reports of a ~25% tissue recovery, 25–60% insulin recovery and a 2–6 times increase of the ratio of insulin to amylase, for large mammal islet isolation methods using extensive digestion or dispersion procedures [13,14,17,19,22]. Although the selective survival of islets in the latter studies have been considered an important contribution to the transplantability of dispersed pancreatic tissue, these

studies in fact demonstrated recovery of acinar tissue to be a more sensitive indicator for excessive trauma than islet recovery. Similar findings have been reported in a histological study on the effects of chopping and collagenase digestion of the canine pancreas by Schwartz et al. [32]. Thus our findings demonstrated that by avoiding traumatic methods the integrity of dispersed tissue can be preserved. A major methodological advance was the introduction in 1989 of the UWS for tissue preservation during the cold phase of islet isolation [37,38]. The near complete purity of the density separated islets, corroborated these preliminary reports of a dramatic improved purity, when the traditionally used physiological salt solutions are replaced by the UWS to prevent cell swelling during isolation. Tentatively, isolation in UWS may likewise have contributed to preservation of the insulin and amylase content of the isolated islet and acinar cells by preventing an increase of the permeability of the cell membrane during hypothermic isolation [35,39].

Little is known about the effects of islet isolation related procedures on the integrity of islets [26]. In contrast to the more compact spheroid form of rodent islets, the irregular form of native large mammal islets precludes qualitative morphological examination of islet integrity after isolation. Morphometric analysis of islet integrity has been hampered by lack of reference values for the normal size and number of islets in the large mammalian pancreas. A high proportion of small islets after isolation has generally been considered a sign for islet fragmentation. In our study, however, comparison of the size distributions of isolated and native islets demonstrated a similar high proportion of small islets and no signs of fragmentation as judged by the largest and average diameter. Diameters in our study usually ranged from 40 to 300 μm, which corroborates previous findings in canine islet isolation [2]. Smaller islets, usually up to 150 μm, were obtained by the Miami group [3,16] from beagle dogs weighing 6–13 kg. The isolation of smaller islets by the Miami group may probably be explained from the lower body wt of their animals, because the maximum diameter of isolated islets correlated with body wt in our study, and similar findings have been reported in studies of the rat [11,24] and dog pancreas [29]. Comparison of 'the number of small islets or fragments' and numerical size distributions have been helpful to study islet integrity during isolation in previous studies [4,7,27,28,41,45]. However, parameters of the numerical size distribution, like the average islet diameter (number-average diameter) and number of small islets, largely depend on the lower limit set to sizing, and thus suffer from the same difficulties, previously encountered when reporting the total number of isolated islets to document islet yield [26,30]. Total islet volume is considered the best parameter to document islet yield.

Because the number and size of larger islets — which mainly determine both the total islet volume and the volume-average diameter — are accurately measured, the volume-average diameter should be considered a more practical and objective parameter to document islet size and integrity, than parameters of the number size distribution.

Assessment of viability by perifusion demonstrated a low basal insulin secretion, indicating well preserved islets, and a tenfold insulin release during physiological glucose stimulation. Our data compare favourably with previous in vitro studies of isolated canine islets, reporting a two to tenfold higher basal insulin release [23,40,41] and a one to fourfold increase over basal insulin secretion during high-glucose perifusion [41] or static incubation [16,23,34]. Virtually no response, at all, to high-glucose has been reported in other studies of isolated canine islets [31,44] and low responses, from 0.5–2 times basal release, were reported for pig islets [4,20,25]. Both an improved preservation of islets by isolation in UWS and the virtual complete absence of acinar tissue in our preparations may have contributed to the excellent in vitro function.

We conclude that the variability of islet and insulin yield may be attributed to a large extent to the variability of the native endocrine pancreas. Isolation efficacy was best documented by morphometry of the isolated and native islet population — which demonstrated a yield of 50% of the islets from the dog pancreas and no fragmentation of islets during isolation by the gentle single-endpoint collagenase digestion technique. Although insulin extraction does not discriminate between free and entrapped islets, assessment by both morphometry and extraction allowed the quantitation of entrapped islets, and demonstrated preservation of ß-cell granulation during isolation. We suggest that similar studies should be done in man to facilitate the analysis of other factors affecting the outcome of islet isolation for transplantation.

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