Automated Method for Isolation of Human Pancreatic Islets

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We describe an automated method for the isolation of human pancreatic islets. The procedure meets the following requirements: *1*) minimal traumatic action on the islets, *2*) continuous digestion in which the islets that are progressively liberated can be saved from further enzymatic action, *3*) minimal human intervention in the digestion process, and *4*) high yield and purity of the isolated islets. After purification on Ficoll gradients, an average of 164,600 islets/pancreas was obtained (2279 islets/g), with an average purity of 78.5% islets. The average volume and average insulin content of the final islet preparation were 348 mm³ and 93.4 U, respectively. The islets were morphologically intact with a normal degree of β-granulation and responded to glucose stimulation with a fivefold increase of insulin secretion over basal levels. The procedure is now being used for the initiation of the second phase of clinical trials on human islet transplants. *Diabetes* 37:413–20, 1988

The pancreas of large mammals differs considerably from that of rodents. Structure, size, and shape of the islets and their attachment to the surrounding exocrine tissue are only some of the variables that make islet isolation unique and different for each species. Even within a species, there can be significant differences in age, volume, and condition of the exocrine pancreas that affect the outcome of the islet isolation procedure. It is therefore easy to understand how the collagenase technique developed for isolation of rodent islets was not effective for mass isolation of islets from large mammals, including humans (1).

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Horaguchi and Merrell (2) developed a method for perfusing the dog pancreas with collagenase via the pancreatic duct. This procedure permitted the isolation of many islets from the canine pancreas. Noel et al. (3) modified this procedure further and developed a method that allows the separation of massive numbers of canine islets. Long et al. (4) utilized a screen method for separating canine islets, whereas Lacy et al. (5) introduced Velcro to retain the partially digested collagen during the isolation procedure in beef and dog pancreases. Also, pig islets have recently been isolated in large numbers with a modification of the collagenase technique that involved a tissue macerator for the mechanical dispersion of the digested tissue (6).

The human pancreas required other modifications of the isolation procedure to obtain high yields of islets. Gray et al. (7) injected collagenase solution into the human pancreatic duct and, after incubation at 37°C, disrupted the digested tissue by shaking and aspiration through different-sized needles. The islets were then separated on Ficoll gradients similar to what had been used for the purification of rat islets (8). The average yield was ~1000 islets/g pancreas, with a purity of 10–40% islets. Kneteman and Rajotte (9) perfused the human pancreas through the duct in a procedure similar to the method of Horaguchi and Merrell (2) for the isolation of canine islets. The digested tissue was teased apart and filtered with partial purification of the islet preparation. The procedure allowed the separation of ~85,000 islets/pancreas, with an average purity of <30% islets. Kuhn et al. (10), after ductal pancreatic perfusion with collagenase, used Velcro to retain the partially digested human pancreas, similar to the method for beef islet isolation (5), with a yield of 80,000 islets/pancreas.

No information was reported concerning purity and viability of the human islets. Scharp et al. (11) developed a distension method for the isolation of human islets in which, after collagenase digestion of the pancreas, the islets were liberated by passage of the predigested pancreas through a tissue macerator. The purification of the islet preparation was initially done with an elutriator, but the purity was only 20–30% islets. Subsequently, Ficoll and Percoll gradients

were used on preparations obtained by the distension method, resulting in purification of the preparation (60–90% islets) with an average islet yield of ~125,000 islets/pancreas after Ficoll purification, representing 2180 ± 325 islets/g pancreas (11). Alejandro et al. (12) have modified the procedure they developed in the dog to isolate many human islets. Alderson et al. (13) combined the perfusion method (9) with the disruption of the pancreas through a tissue macerator (11) for the isolation of human islets from young donors. An average of 138,000 islets was obtained before purification with Ficoll. After Ficoll, the purity of the final preparation was ~50% islets. Rajotte et al. (14) compared the distension technique with the perfusion technique, obtaining an average of ~120,000 unpurified islets/human pancreas with the perfusion method.

All procedures developed for the isolation of islets from large mammals including humans have in common a significant traumatic factor in the isolation process; chopping, teasing, aspiration through needles, shaking, and passage through a tissue macerator are all methods that can easily break the islets. In addition, the digestion or predigestion process is generally stopped when subjective observation of samples reveals an estimated optimal digestion, and the isolation process is then continued by other means. At the critical moment when the pancreatic digestion is stopped, part of the pancreas is generally still underdigested, and other portions are overdigested. What is saved is the portion of the preparation that at that moment is optimally dissociated, when the islets are liberated from the exocrine pancreas but before they become damaged from the collagenase activity. Scharp et al. (15,16) developed a digestion-filtration method that permitted complete digestion of the pancreas while removing the islets from the digestion media; however, the method was not effective for isolation of human islets.

We have studied another approach to islet isolation that could meet the following requirements: *1*) minimal traumatic action on the islets, *2*) continuous digestion in which the islets that are progressively liberated can be saved from any further enzymatic action, and 3) minimal human intervention in the digestion process.

The result is an automated method in which the pancreas is digested after distension with a collagenase solution in an isolator (an isolation device we developed) that allows the progressive liberation of the islets from the gland. At the end of the digestion process only a fibrous network of ducts and vessels remains in the isolator, indicating that the pancreatic digestion with this method is complete, with no waste of pancreatic tissue.

After initial promising results with pancreases from pigs and other mammals (unpublished data), this method has been tested on the human pancreas. This report describes an automated procedure for the isolation of human islets and the functional and morphological status of the isolated islets.

MATERIALS AND METHODS

Islet isolation procedure. The human pancreases were obtained from the network developed by the National Diabetes Research Interchange (NDRI). Once the pancreas has been removed from the deceased donor, it is placed in Eurocollins solution (Fresenius, Hamburg, FRG) at 4°C. The gland may be stored in this manner for several hours, although the best results were obtained with glands removed <18 h before the isolation procedure. The pancreas was trimmed by careful dissection of the surrounding fat tissue, lymph nodes, vessels, and membranes.

The pancreatic duct was cannulated with an 18-gauge angiocatheter. Hanks' balanced salt solution (Sigma, St. Louis, MO) containing 2% fetal calf serum and 2 mg/ml collagenase (Sigma, type X) was injected through the pancreatic duct (Fig. 1). The amount of collagenase solution injected was approximately equal to double the pancreas weight at 28–30°C.

The pancreas was then loaded into the isolator (Fig. 2), which consists of two stainless steel chambers (lower and upper) separated by a stainless steel screen (280-μm mesh). The total inner volume of the isolator is 300 ml. The lower cylindrical chamber has two inlet ports and a third opening on the side for a temperature probe. Seven glass marbles (1 cm diam) are placed in the chamber. The upper chamber is conical and has one output opening. The two portions of the isolator are held together by three screws (Fig. 2), and the device is connected to a shaker (model 75, Burrel, Pittsburgh, PA) by a stainless steel bar. Once the gland is loaded into the isolator, additional collagenase solution (2 mg/ml)

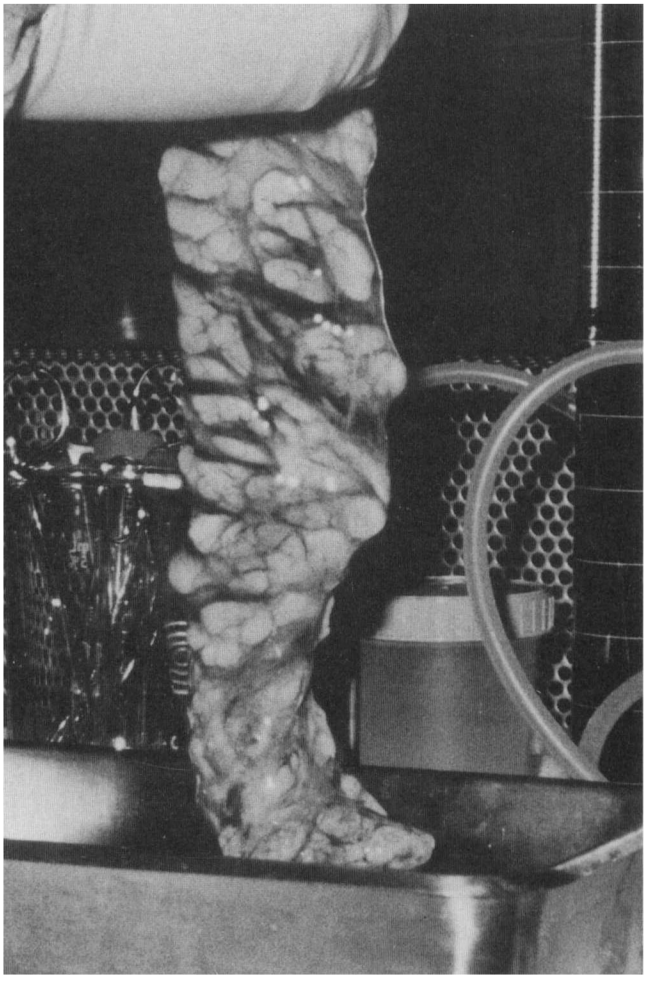

FIG. 1. Human pancreas after intraductal injection of collagenase solution. Gland is loaded into isolator (Fig. 2) in 1 piece.

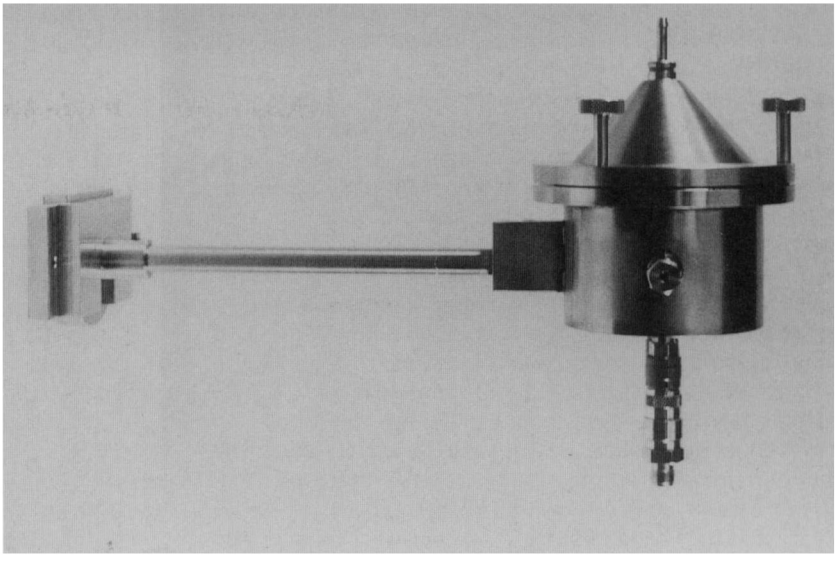

FIG. 2. Stainless steel isolation chamber (isolator) constructed for digestion of human pancreas.

is added to fill the lower chamber. The screen is placed in position, and the device is closed by connecting the upper to the lower chamber, which is sealed by an O-ring. The shaker is activated (320 oscillations/min), and the peristaltic pump is turned on with a flow rate of 40 ml/min. At this stage the digestion is conducted in a closed system in which Hanks' solution is aspirated through a filter (94-μm mesh) placed in the upper portion of the recirculation cylinder (Fig. 3). From the recirculation cylinder, the solution passes through the pump to a heating circuit and reaches the lower chamber of the isolator, progressively diluting the collagenase solution inside the chamber. The heating circuit allows the progressive increase of the temperature in the digestion chamber (1°C/min) until 37°C is reached. Then a heating circuit bypass is switched on and off to maintain a stable temperature in the digestion chamber.

From the chamber, the solution passes through the 280-μm screen to a cooling circuit that inactivates the collagenase and then comes back to the lower portion of the recirculation cylinder. Both the cooling circuit and the recirculation cylinder are placed in an ice bath (4°C), which inactivates the collagenase and preserves the islets. The

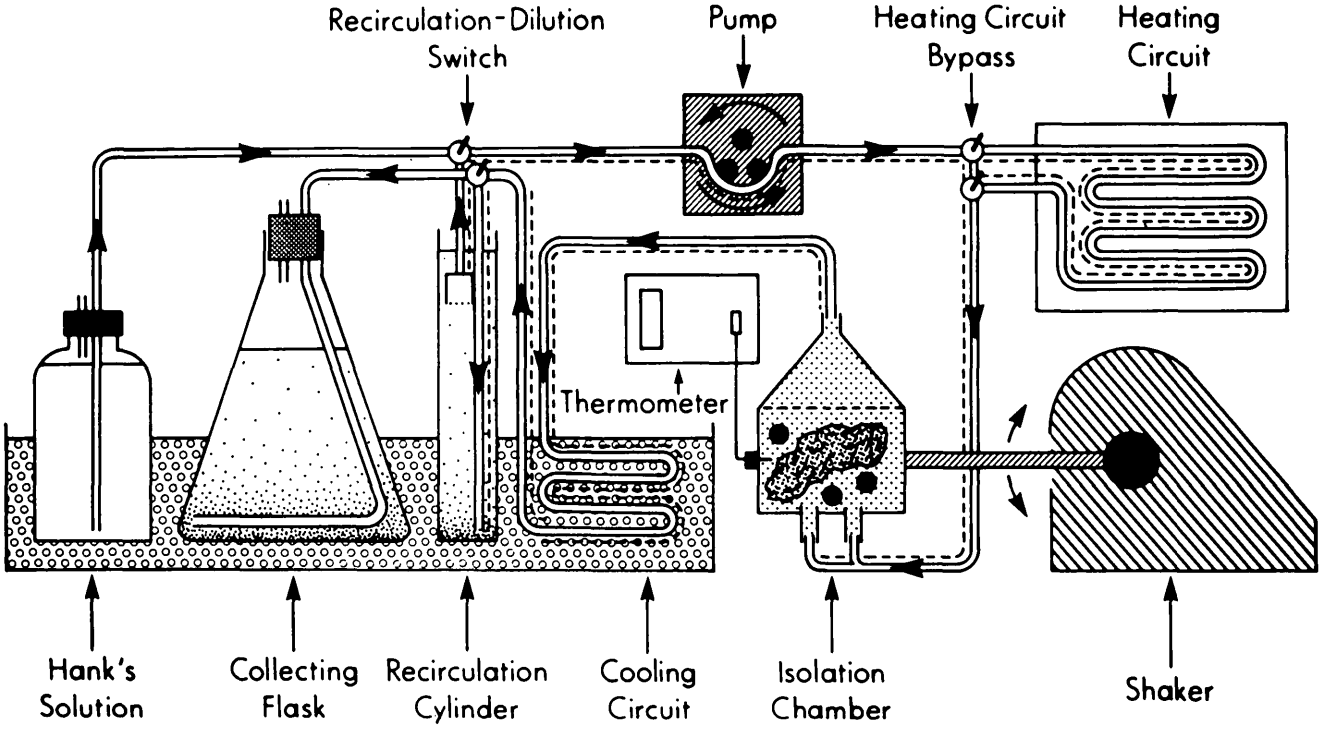

FIG. 3. Automated procedure for isolation of human pancreatic islets.

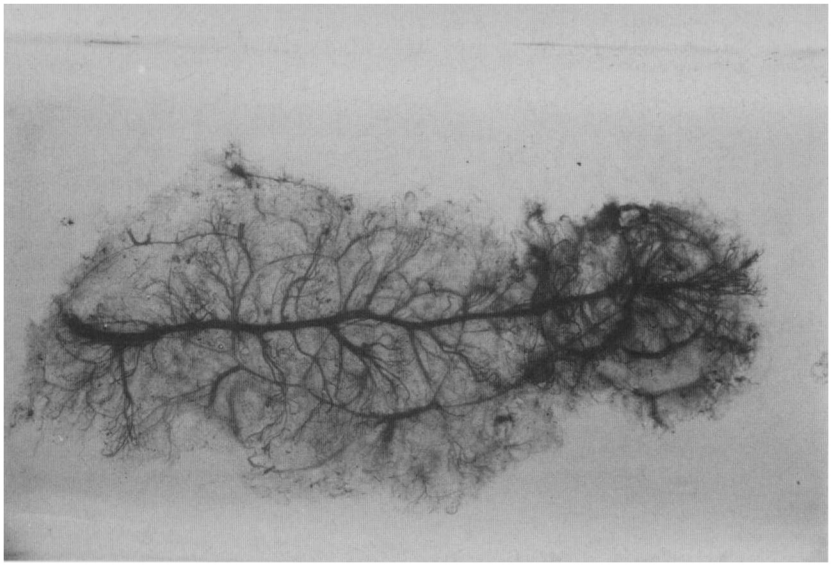

FIG. 4. Fibrous network of ducts and vessels that remains in isolator after digestion of human pancreas (tail and body). Integrity of small branches indicates minimal traumatic action of isolation procedure.

solution is drawn from the top of the recirculation cylinder through the 94-μm filter while the islets remain settled at the bottom of the cylinder.

Samples of the preparation are taken every 2 min from a stopcock near the output opening of the digestion chamber to monitor the digestion. When free islets are detected in a sample, the recirculation cylinder and the heating circuit are bypassed and the islet separation is conducted on an open system in which the temperature progressively decreases and the collagenase solution is diluted by fresh Hanks' solution. The islet preparation is then collected in 2-L flasks. In the open-system phase the flow rate increases up to 200 ml/min, allowing a faster turnover of the digestion chamber content. The isolation procedure is generally terminated after 30–60 min, when islets are no longer detected in the samples. At this time, only a fibrous network of ducts and vessels remains in the digestion chamber (Fig. 4).

Purification of islet preparation. The purification of the islet preparation was accomplished by centrifugation on Ficoll gradients similar to the method described previously (11). Briefly, 3-ml aliquots of pelleted tissue from the islet preparation were loaded into 250-ml plastic syringes in the following manner. One hundred milliliters of a Ficoll (lyophylized and dialyzed, Sigma) gradient with a density of 1.074 g/cm^3 were pumped into each syringe. The aliquots were suspended in 10 ml of Hanks' solution, added to 40 ml of a Ficoll gradient (density = 1.058 g/cm^3), and layered over the 1.074-g/cm^3 gradient. The syringes were then centrifuged at 800 × *g* for 16 min at 4°C. The top layer (20–30 ml) containing cells and fragments was discarded; the next 50–80 ml of the gradient contained the purified islets (Fig. 5). The acinar tissue was pelleted in the bottom of the syringe (Fig. 6). If the separation is optimal, only a few islets should appear in the acinar pellet.

The layer containing purified islets was washed with Hanks' solution by centrifugation at 950 × *g* for 5 min at 4°C. The islet pellets were combined and suspended in 200 ml of tissue culture medium (CMRL-1066) containing 10% fetal

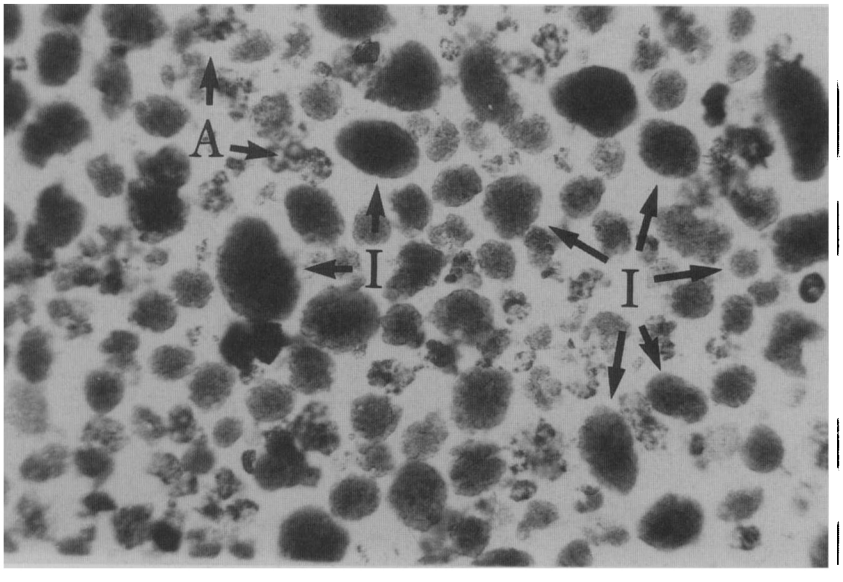

FIG. 5. Phase photomicrograph of purified human islet preparation immediately after isolation. Islets (*I*) are clearly distinguished from acinar tissue (*A*). ×20.

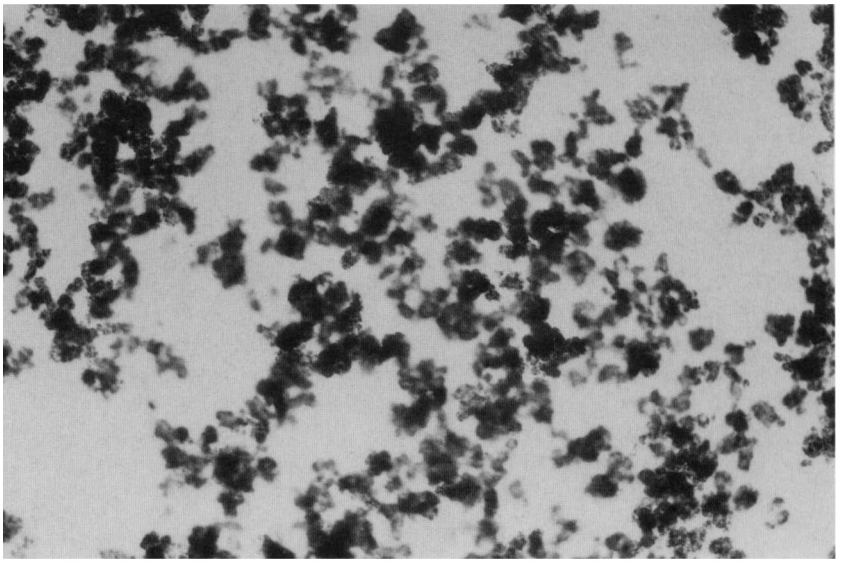

FIG. 6. Phase photomicrograph of acinar tissue that settled in bottom layer after centrifugation with Ficoll gradients while purified islets were saved in upper layers. ×20.

calf serum, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), D-glucose (1 mg/ml), L-glutamine (2 mM), and HEPES (25 mM) and were adjusted to pH 7.4 with NaOH. An 0.5-ml aliquot was removed to determine total islet count, islet mass, and the purity of the preparation with a phase microscope fitted with a green filter. With phase microscopy the islets appear as dark brown round structures and can be distinguished easily from the acinar tissue (Figs. 5–8). A calibrated grid in the eyepiece was used to determine the islet volume. The islets were divided into four diameter classes: 50–100, 100–200, 200–300, and >300 μm. Islet tissue smaller than 50 μm in diameter was not considered in the count. The islets were considered as spheres for determination of volume. The purity of the preparation was estimated to be 65–95% islets.

In vitro perifusion. The functional integrity of the islets contained in the final preparation was determined by in vitro perifusion of the islets after 1 day of culture (11). After the purification process, the islets were cultured overnight at 37°C in untreated T-flasks to prevent their adherence. Each flask contained 6000 islets in 50 ml of CMRL-1066. The in vitro perifusion method (11) was used to test insulin secretory activity from an aliquot of the islet preparation equivalent to 100 islets with an average diameter of 150 μm. The chambers contained a cellulose acetate Millipore filter (8 μm), and the islets were perifused at 37°C with Krebs-Ringer bicarbonate solution containing 0.5% albumin and oxygenated with 95% O_2/5% CO_2. The islets were perifused with glucose concentrations of 60, 300, and 60 mg/ml for 40 min at each concentration. The flow rate was 1 ml/min, and the perifusate was collected with an automatic fraction collector. The samples were frozen for subsequent insulin assay, which was performed by the Diabetes Research and Training Center (DRTC) Radioimmunoassay Core Facility.

Insulin extraction. Triplicate aliquots of the final preparation equivalent to 100 islets were taken the day of the isolation.

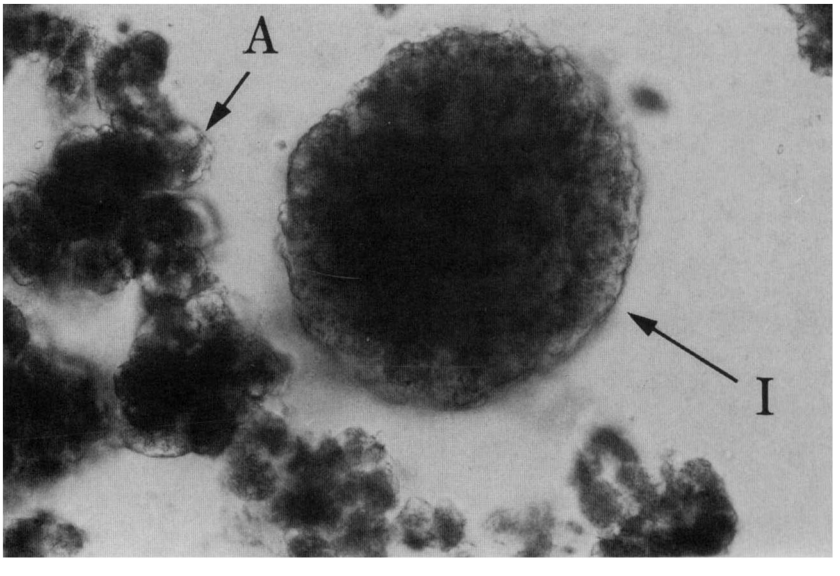

FIG. 7. High-magnification phase photomicrograph of human islet from sample during isolation procedure before purification with Ficoll gradients. Regularly shaped islet (*I*) is easily distinguished from surrounding acinar tissue (*A*). ×100.

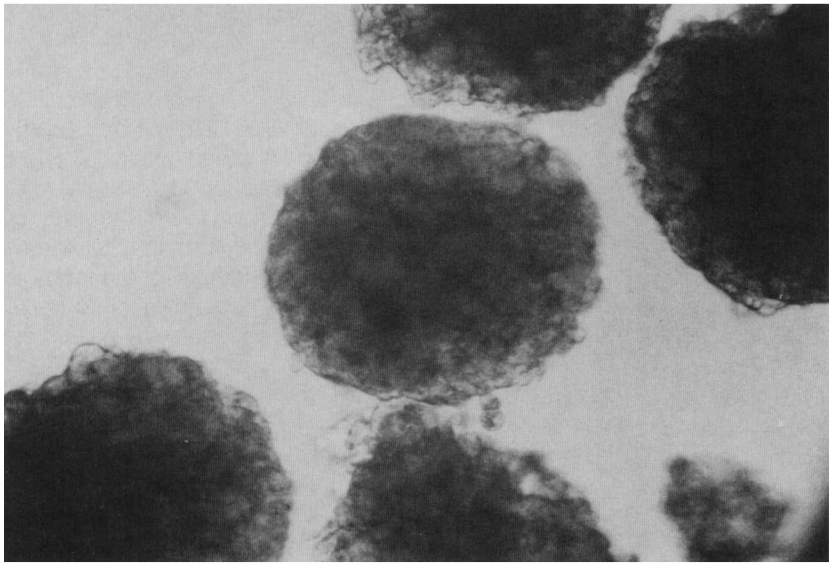

FIG. 8. High-magnification phase photomicrograph of purified human islets immediately after isolation. ×100.

The islets were centrifuged, the supernatant discarded, and the pellet resuspended with 1 ml acid alcohol for insulin extraction. After overnight incubation at 4°C, the extracts were diluted and frozen for subsequent insulin assay.

Histology of islets. Aliquots of the final islet preparation were pelleted in a microfuge tube by centrifugation, fixed in Bouin's, processed for light microscopy, and stained with aldehyde fuchsin and hematoxylin and eosin. The histologic preparations were done by the DRTC Histology Core Facility.

RESULTS

The results from 10 different isolations are summarized in Table 1. Before the purification process the isolation procedure allowed the separation of an average of 275,800 islets/pancreas with an estimated volume of 818 mm^3. After Ficoll gradients the average purity of the islet preparation was 79% islets. The variability in exocrine tissue present in the final preparation was due to the condition of the pancreas at the time of the isolation procedure. If the acinar tissue is degranulated, it becomes less dense and does not settle in the bottom layer during the centrifugation with Ficoll gradients. After the purification process, an average of 164,600 islets/pancreas (348 mm^3 average islet volume) was obtained, representing an average of 2279 islets/g pancreatic tissue.

The insulin secretory response of an aliquot from the final islet preparation after Ficoll purification and overnight culture is shown in Fig. 9. Islets from nine different isolations were cultured overnight at 37°C and then perifused in the presence of 60, 300, and 60 mg/dl glucose for 40 min at each glucose concentration. The flow rate was 1 ml/min, and samples for insulin determination were collected every 5 min. The results are expressed as increments from the average basal insulin secretion of the 15 min before stimulation. An approximately fivefold average increase from the basal insulin secretion was observed. The secretory response was maintained for all of the 40 min of glucose stimulation (300 mg/dl) and returned to basal levels after perifusion with the

TABLE 1
Automated method for isolation of human islets

	Pancreas data				Prepurification			Postpurification			Recovery			
Isolation no.	Donor age (yr)	Pancreas weight (g)	WIT (min)	CIT (h)	Total no. (×1000)	Total volume (mm^3)	Islets/g	Total no. (×1000)	Total volume (mm^3)	Islets/g	Islet no. (%)	Islet volume (%)	Purity (% islets)	Insulin extraction (U)
1	24	56	14	20	267.0	458	4768	150.9	125	2693	57	27	50	87.9
2	29	60	0	14	326.7	632	5433	128.5	287	2142	39	45	75	74.4
3	26	58	0	6	245.8	647	4237	164.8	284	2841	67	44	90	85.2
4	28	58	9	20	135.6	398	2338	74.4	96	1283	55	24	75	72.9
5	40	57	0	15	449.4	1537	7884	227.2	504	3986	51	32	95	106.7
6	45	126	0	14	511.5	1930	4059	330.4	1022	2622	65	53	80	201.0
7	57	86	0	21	171.5	435	1994	90.4	102	1051	53	23	75	Cryopreserved
8	28	64	0	6	161.0	571	2516	97.6	265	1525	61	47	95	100.1
9	47	84	0	18	233.4	805	2779	135.6	354	1614	58	44	55	66.2
10	38	81	0	13	255.8	765	3158	246.0	436	3037	96	57	95	46.4
Mean	36.2	73	2.3	14.7	275.8	818	3917	164.6	348	2279	60.0	39.6	78.5	93.4
SD	11.0	22.1	5.0	5.4	122.6	509.2	1792.2	80.6	273.8	920.3	14.8	12.2	16.2	51.1
SE	3.5	7.0	1.6	1.7	38.8	161	567	25.5	87	291	4.7	3.9	5.1	14.7

Results are from 10 different isolations. WIT, warm ischemia time; CIT, cold ischemia time.

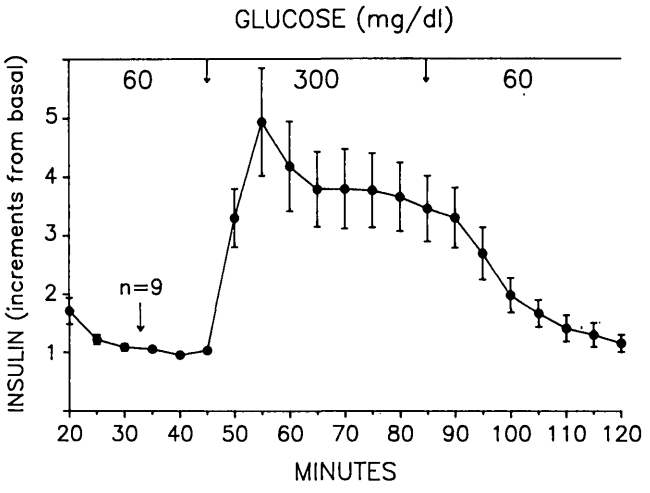

FIG. 9. Perifusion of purified human islets after 1 day of in vitro culture at 37°C. Insulin secretion is represented as increments from average basal secretion of the 15 min before stimulation (assumed to be 1).

low-glucose solution (60 mg/ml). A lag period of 6 min occurred in the perifusion system due to the dead space in the tubing that retarded the arrival of the solutions to the perifusion chamber. This finding indicates that human islets isolated by this method were viable and functionally intact 1 day after the isolation and purification procedure.

The insulin content of the purified islets was determined at the time of the perifusion from an aliquot of the final preparation (Table 1) and resulted in an average of 93.4 U insulin/isolation. Histological studies of the islet preparations showed intact islets with normal degree of β-granulation after the isolation procedure (Fig. 10).

DISCUSSION

The isolation procedure described by Gray et al. (7) allowed the separation of an average of <40,000 islets/human pancreas, representing 1101 ± 450 islets/g pancreas, with an average purity of the final preparation of 28% islets. Our laboratory developed a distension method that improved the isolation procedure both in yield and purity of the final islet preparation (11). An average of ~125,000 islets/pancreas was obtained, with a purity of the final preparation of 60–90% islets (after Ficoll gradient purification). The final islet volume was 199.4 ± 40.9 mm^3. One limitation of the distension method was that the donor age had to be >27 yr, with best results obtained in pancreases from donors >40 yr old.

The automated method described in this paper produced minimal traumatic action on the islets with complete digestion of the pancreas. As a result of progressive digestion, the whole pancreas is gently disrupted. A residual network of vessels and ducts with small branches remains in the digestion chamber at the end of the isolation process (Fig. 4), indicating that the method digests the pancreas with minimal traumatic action to the islets.

The automated method produced an improvement both in average purified islet yield and islet volume (164,600 islets, 348 mm^3) in comparison with the distension method previously developed in our laboratories (125,700 islets, 199 mm^3). The islet yield increased ~30%, and the islet volume was 75% higher than with the distension method, suggesting that the automated method is less traumatic and the islets are less likely to be broken into smaller fragments during the isolation procedure.

The average insulin content was 93.4 U/isolation, representing a 78% increase in insulin yield in comparison with the distension method (52.4 U/isolation, $P < .05$).

The purified islets were morphologically intact at the time of the isolation (Fig. 10) and responded to glucose stimulation after overnight culture at 37°C, with a fivefold increase from the basal insulin secretion (Fig. 9).

Another advantage is that the procedure works effectively on pancreases from donors of different ages (24–57 yr). Pancreases from donors under the age of 27–30 yr are generally digested in a shorter time, whereas older ones require a longer digestion period. The procedure for digesting the pancreas remains essentially the same, because the procedure is monitored through the sampling of the effluent from the digestion chamber. If the pancreas is younger, with less collagen content, it will fall apart in a shorter time, and the

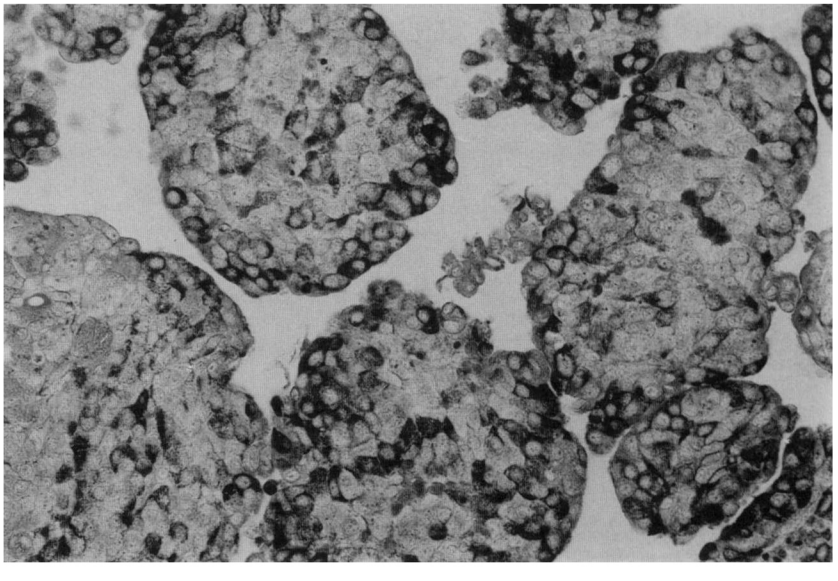

FIG. 10. Photomicrograph of purified islet preparation immediately after isolation. β-Cells have normal degree of granulation. Aldehyde fuchsin stain. ×160.

collagenase will be diluted earlier with the cold solution in the open-system phase.

The results reported in this study are encouraging enough to believe that they will fulfill the minimal estimates required for an islet transplant in humans (11). The automated method for the isolation of human islets and the Ficoll purification procedure are now being used for the initiation of the second phase of clinical trials of islet transplantation in patients with insulin-dependent diabetes mellitus.

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