Normalization of Diabetes in Spontaneously Diabetic Cynomologus Monkeys by Xenografts of Microencapsulated Porcine Islets without Immunosuppression

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Abstract

Porcine pancreatic islets were microencapsulated in alginate-polylysine-alginate capsules and transplanted intraperitoneally into nine spontaneously diabetic monkeys. After one, two, or three transplants of 3–7 $\times$ 10^4 islets per recipient, seven of the monkeys became insulin independent for periods ranging from 120 to 804 d with fasting blood glucose levels in the normoglycemic range. Glucose clearance rates in the transplant recipients were significantly higher than before the graft administration and the insulin secretion during glucose tolerance tests was significantly higher compared with pretransplant tests. Porcine C-peptide was detected in all transplant recipients throughout their period of normoglycemia while none was found before the graft administration. Hemoglobin A_{1C} levels dropped significantly within 2 mo after transplantation. While ketones were detected in the urine of all recipients before the graft administration, all experimental animals became ketone free 2 wk after transplantation. Capsules recovered from two recipients 3 mo after the restoration of normoglycemia were found physically intact with enclosed islets clearly visible. The capsules were free of cellular overgrowth. Examination of internal organs of two of the animals involved in our transplantation studies for the duration of 2 yr revealed no untoward effect of the extended presence of the microcapsules. (*J. Clin. Invest.* 1996. 98:1417–1422.) Key words: pancreatic islets • microencapsulation • xenotransplantation • nonimmunosuppression

Introduction

Islet transplantation has been slow to develop as a therapy for type 1 diabetes mellitus (1–5). Two major causes for the high incidence of failure in human clinical allotransplantations can be identified: (*a*) the poor availability of transplanted pancreatic tissue; and (*b*) the need for permanent immunosuppression. The development of techniques which would make it possible to transplant nonhuman pancreatic islets in the absence of immunosuppression clearly represents the most favored solution.

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To curtail immunorejection we have developed alginate-polylysine-alginate biocompatible capsules to enclose individual pancreatic islets. In our earlier studies both allo- and xenografts of microencapsulated islets were shown to reverse diabetes in long-term experiments in spontaneously diabetic as well as chemically induced diabetic rodents (6–10). Recently, we have developed a method for isolation of porcine islets of high quality and quantity (11).

Here we report the results of a preclinical study in which microencapsulated porcine islets were transplanted into spontaneously diabetic cynomologus monkeys.

Methods

Islet isolation. The pancreata were obtained from large sows of a well-defined genetic background (Yorkshire $\times$ Landrace) weighing in excess of 200 kg each with a warm ischemia time of $<$ 5 min. Porcine islets of Langerhans were isolated by a modification of the method previously described by our group (11). In the modified method, significantly lower concentrations of collagenase (Boehringer-Mannheim, Mannheim, Germany), 0.6–0.8 mg/ml, were used. In the procedure, a cannula was inserted in the common duct and a solution of collagenase P in modified Hank's solution (2 ml/1 gram of tissue) was perfused through the gland. The perfused pancreas was gently shaken and digested at 37°C for 20–24 min. The digested tissue was then passed through a 300-μm screen to remove any remaining larger pieces and centrifuged at 200 *g* for 3 min. The sediment was then centrifuged on dextran (Sigma Immunochemicals, St. Louis, MO) density gradients at 700 *g* for 10 min. Debris was removed from islet suspension by passage through a 50-μm screen. The procedure results in islets retaining their original compact, wholesome appearance with reduced tendency to fragment. The islet yields averaged 2,124±723 islets/gram of pancreas. Due to the higher compactness of the isolated islets, the islet purification on dextran density gradients results in islets of a high degree of purity, $>$ 95%. (Before the islet isolation method was improved to the point of attaining this degree of purity, islet isolates only 75–90% pure could be produced. As described later in this manuscript, these less pure islets resulted in only a partial lowering of diabetic hyperglycemia after transplantation into two diabetic monkeys.) In addition, the purified islets possess a higher degree of viability as compared with islets isolated with higher enzyme concentrations. After the isolation, islets of 50–300 μm in diameter and of $>$ 95% purity were used for encapsulation. Purity is assessed by counting 10 microscopic fields usually containing 300–500 islets and establishing the number of islets as well as the number of pieces of the contaminating acinar tissue debris. Dithiazone (diphenylthio-carbazone) staining was used throughout this study to identify and quantify endocrine pancreatic tissues. The viability and the physiological functionality of isolated islets was measured in vitro in a static glucose challenge, evaluating the ability of the islets to secrete insulin in response to different glucose concentrations. The exposure of islets to the high glucose concentration (16.5 mM) which results in at least double the amount of insulin secretion compared with the low glucose (2.7 mM) is considered a positive response.

Islet encapsulation. The encapsulation technique was a modification of Sun's method (12). The modification involved the use of an electrostatic droplet generator (8), which produces smaller, stronger, and more uniform capsules compared with the older air-jet tech-

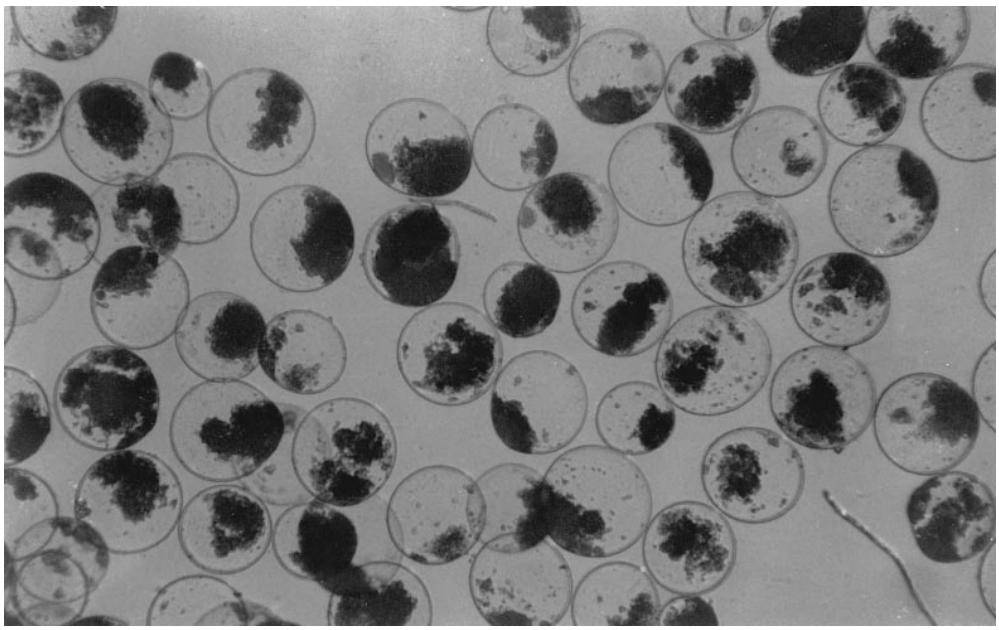

Figure 1. Micrograph of porcine islets microencapsulated in alginate-polylysine-alginate membranes. ×35.

nique. The islets were suspended in 1.5% (wt/vol) purified sodium alginate (Kelco Gel LV; Kelco, San Diego, CA) at a concentration of ~ 3,000 islets/ml. Spherical droplets were formed by the electrostatic field interaction coupled with syringe pump extrusion and were collected in a 100 mM calcium lactate solution. The gelled droplets were washed with 0.9% saline before suspension in 0.05% poly-L-lysine (Sigma, 22,000–24,000 D) for 5 min. The droplets were again washed with 0.9% saline, the capsules were allowed to react with 55 mM sodium citrate for 4 min and finally washed with 0.9% saline and with culture medium. Most capsules contained one islet and had a diameter of 0.25–0.35 mm (Fig. 1).

Islet transplantation and xenograft evaluation. The transplant recipients were spontaneously diabetic monkeys obtained from a primate breeding colony (Health and Welfare Canada, Ottawa, Ontario, Canada), from the Hazelton Laboratories (Madison, WI), and from the California Regional Primate Research Center (Davis, CA), 3.2–5.6 kg of body weight. The animals had developed diabetes in captivity between 3 and 12 yr of age. Nine diabetic monkeys were transplanted with microencapsulated islets, while an additional two animals received grafts of free, unencapsulated islets. The islet grafts were delivered by intraperitoneal injection under light ketamine anesthesia. In each transplantation procedure 30,000–70,000 encapsulated islets were delivered. Each monkey received one, two, or three transplants, 2–3 wk apart. Blood glucose monitoring was performed daily. Amex Dextrostix and Ames Glucometer as well as a glucose analyzer (Beckman Instruments Inc., Fullerton, CA) were used for glucose determination. The insulin content in the serum was determined by radioimmunoassay (Coat-a-Count, Diagnostic Product Corp., Los Angeles, CA). Intravenous glucose tolerance tests (IV GTT,[1] 0.5 grams glucose/1 kg body weight) were administered before the transplants and at monthly intervals after the graft administration. Porcine C-peptide presence in the sera of the recipient animals was tested before and after transplantation, in 4–6-wk intervals, using a radioimmunoassay kit (Linco Research Inc., St. Louis, MO). Glycosylated hemoglobin concentrations were measured in the blood of the experimental animals before the graft administration, 2 mo after transplantation, and every 3 mo thereafter using commercially available diagnostic kits (Biorad, Hercules, CA). The presence of antiislet antibodies was established in the sera of the transplant recipients by an indirect immunofluorescence assay. To study the condition of the transplanted capsules a small portion of microencapsulated islets was recovered from two normoglycemic transplant recipients 3 mo after transplantation. The operation was performed under ketamine anesthesia. Subsequently, some of the recovered microcapsules were cultured in RPMI 1640 tissue culture medium, supplemented with 100 mg% glucose and 7.5% calf serum to evaluate in vitro in a static glucose challenge the viability and physiological competence of the encapsulated islets.

Histology. After the conclusion of the study, two of the experimental animals (Nos. 3 and 6) were killed and their pancreata were removed and fixed in Bouin's solution for ~ 4 h. Thin sections were then prepared and stained with aldehyde-fuchsin. In addition, immunostaining for insulin and glucagon using the peroxidase method was also performed.

Results

All of the diabetic animals displayed clinical features of type 1 diabetes mellitus including polyuria, polydipsia, polyphagia,

Table I. Variation of Daily Insulin Needs in the Graft Recipients before Transplantation

Monkey	Variation of daily insulin needs
1	6–10
2	3–6
3	6–11
4	4–9
5	6–9
6	3–5
7	4–8
8	4–9
9	7–11

1. *Abbreviation used in this paper:* IV GTT, intravenous glucose tolerance test.

Table II. Diabetic Control of Insulin-independent Graft Recipients

Monkey	Fasting blood glucose		Hb A_1C		*K* value		Porcine C-peptide		Plasma insulin				Duration of insulin independence	Number of transplants	Total of transplanted islets
									Pre-Tx		Post-Tx				
	Pre-Tx	Post-Tx	Pre-Tx	Post-Tx	Pre-Tx	Post-Tx	Pre-Tx	Post-Tx	Basal	Stimulated	Basal	Stimulated			
	mM		%				*nm/liter*		*μU/ml*				*d*		*islets/kg body wt*
1	17.5	7.9	7.3	4.2	0.25	1.8	0	0.30	1.5	1.4	5.8	25	303	2	18500
2	20.0	7.4	8.0	3.1	0.45	2.2	0	0.25	2.0	2.1	7.0	22	280	2	23300
3	18.0	4.0	6.0	2.0	0.35	2.5	0	0.50	3.0	2.8	5.1	30	250	1	12500
4	15.0	5.9	7.2	3.0	0.30	2.0	0	0.31	3.1	3.1	8.0	32	150	1	9250
5	21.0	7.2	6.3	3.2	0.27	2.5	0	0.28	1.5	1.6	6.5	24	803	2	22000
6	21.0	6.6	9.0	4.5	0.32	1.9	0	0.25	1.0	1.9	6.0	20	123	1	11670
7	19.5	4.5	8.0	3.4	0.51	2.0	0	0.22	3.4	3.0	8.2	25	120	1	15600
Mean±SEM	19.6±3.4	6.2±1.4	7.4±1.0	3.5±0.6	0.35±0.1	2.1±0.3		0.30±0.1	2.4±0.8	2.3±0.7	6.7±1.1	25±4.2	289		16100
P		< 0.05*		< 0.05*		< 0.05*				< 0.05‡		< 0.05‡			

*Compared to Pre-Tx values; ‡Compared to basal values.

weight loss and fatigue, persistent fasting hyperglycemia, persistent glycosuria, and the need for daily injections of insulin. Ketones were detected in the urine of all animals. To normalize the hyperglycemic condition in the diabetic animals, daily doses of insulin ranging from three to eleven international units were administered (Table I). Before transplantation, the fasting blood glucose levels were detected at 19.6±3.4 mM. The glucose clearance rates as determined in IV GTTs and expressed in *K* values ranged from 0.27 to 0.51 (mean 0.35±0.1) before the transplantation. The mean basal pretransplantation insulin levels were at 2.4±0.8 μU/ml. These insulin concentrations remained virtually unchanged during the glucose tolerance tests. Hb A_1C concentrations detected in pretransplant animals were at 7.4±0.1% while the Hb A_1C levels in healthy monkeys were 2.35±0.18%. No porcine C-peptide was detected in the sera of the diabetic animals before the transplantation. Similarly, nondiabetic animals were also negative for the serum C-peptide.

No immunosuppressive therapy of any kind was used throughout this study.

As a result of the engraftment, normoglycemia was restored in seven out of the nine recipient monkeys, with average fasting glucose concentrations of 6.20±1.41 mM. Normoglycemia was established within 2–7 d after the administration of the graft, during which time the exogenous insulin administration was gradually withdrawn. All data pertaining to the diabetic control of insulin-independent graft recipients before and after transplantation are summarized in Table II.

The duration of normoglycemia after the original grafting ranged from 120 to 803 d. The glucose clearance rates in the transplant recipients (*K* values) averaged 2.10±0.3. The insulin secretion as measured in the sera of the transplant recipients in response to IV GTT peaked at 25.4±4.2 μU/ml. This compares with the value of 2.4±0.8 μU/ml during the pretransplantation IV GTT. The porcine C-peptide was detected in all transplant recipients throughout their periods of normoglycemia with concentrations ranging from 0.22 to 0.50 mg/ml. Hb A_1C concentrations declined to 3.5±0.6% 2 mo after transplantation.

All animals were found ketone free during the entire duration of euglycemia. In one animal high concentrations of serum cholesterol (1,430 mg/100 ml) and triglycerides (3,570 mg/100 ml) were detected before the grafting. 1 mo after the grafting the concentrations declined to 260 mg/100 ml and 305 mg/100 ml for cholesterol and triglycerides, respectively.

The sera of all experimental animals were tested repeatedly after the graft administration for the presence of anti–porcine islet antibodies. No antibodies were detected in either of the experimental monkeys, not even after return of hyperglycemia.

The general condition of all recipient monkeys, which had been adversely affected by their diabetic condition before the transplants, improved significantly. While before the initiation of the transplantation program the animals were clearly sluggish and fatigued, they became much more active after euglycemia had been restored. All of the seven animals increased their body weights during the period of normoglycemia. While the average body weight of the diabetic monkeys was 3.1±0.6 kg at the time of transplantation, it increased to 3.8±0.7 kg 3 mo after transplantation. All the clinical features of the human type 1 diabetes displayed by the experimental animals before the graft administration were reversed for the overall duration of normoglycemia.

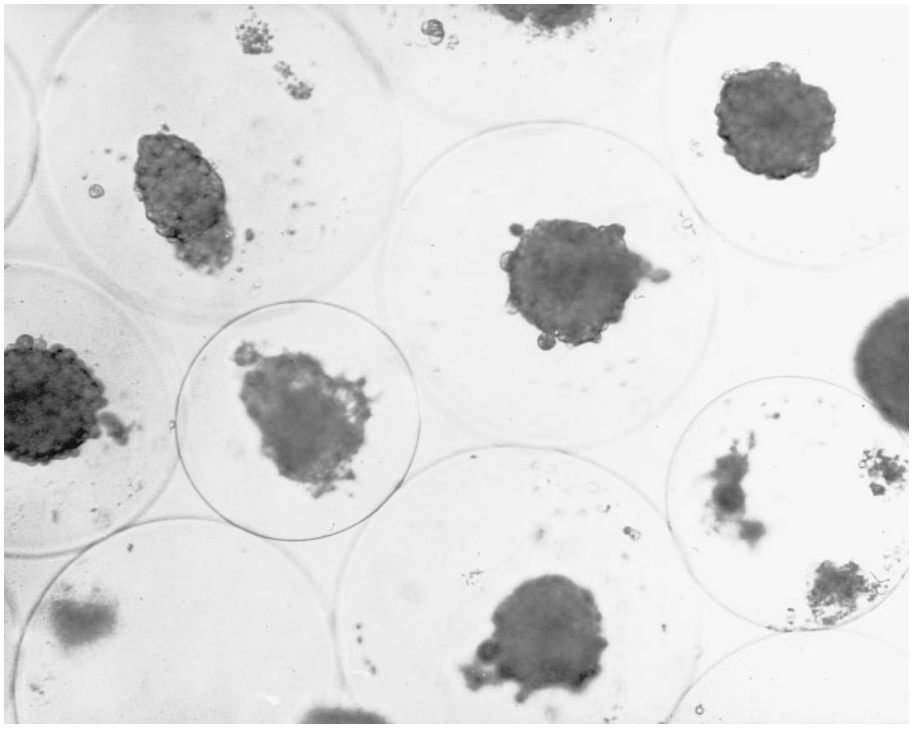

Figure 2. Micrograph of microencapsulated porcine islets recovered from a graft recipient after 3 mo of normoglycemia. ×120.

The reappearance of hyperglycemia 120–804 d after the original series of grafts could again be reversed after the implantation of a similar number of encapsulated islets.

To study the condition of the transplanted capsules a small proportion of microencapsulated islets was recovered from two transplant recipients (Nos. 2 and 5) 3 mo after transplantation. The recovered capsules were free of cell overgrowth and physically intact with the enclosed islets clearly visible. No clumping of the capsules was observed (Fig. 2). In an in vitro static glucose challenge, the secretion of insulin in response to glucose stimulation of the recovered islets was found to be similar to freshly isolated islets. (In a typical experiment involving freshly isolated islets before transplantation, the average insulin secretion in a 24-h static glucose challenge in response to low glucose [2.7 mM] averaged 680±87 pM/24 h/islet, while in response to high glucose [16.5 mM] it rose to 2,671±262 pM/24 h/islet. After islet recovery 3 mo after transplantation, exposure to low glucose in a typical experiment resulted in an average insulin secretion of 593±63 pM/24 h/islet which rose to 2,387±274 h/islet for islets exposed to high glucose.)

Two of the nine recipients never became insulin independent in spite of an additional, third, transplant. Their insulin requirement was significantly reduced to less than half for a considerable period of time, 66 and 153 d for the two animals, respectively (Table III). Examination of retrieved capsules revealed many empty capsules, thus indicating that islet cells had lysed. Since these transplants were performed at the early stages when the purity of the porcine islets was below the 95% level later achieved we can speculate that the contaminating exocrine cells may have caused damage to the encapsulated islet cells.

An additional two control diabetic animals, with pretransplant fasting blood glucose levels of 21.3±0.83 mM, were transplanted with 5.5×10^4 and 7.0×10^4 free, unencapsulated islets, respectively. Euglycemia was achieved within 72 h after transplantation. However, the fasting blood glucose levels started to rise and within 9 d reached the original pretransplant concentrations, thus indicating the destruction of the unprotected graft by the immune system of the host.

The examination of internal organs of two of the animals (Nos. 3 and 6) involved in our transplantation studies revealed no untoward effect of the extended presence of the microcapsules. No adhesions nor any inflammatory processes were detected inside the peritoneal cavity. The liver, kidneys, and the

Table III. Partial Diabetic Control Achieved in Two Graft Recipients

	Fasting blood glucose		Hb A_1C		*K* value		Porcine C-peptide		Plasma insulin (μU/ml)			
									Pre-Tx		Post-Tx	
Monkey	Pre-Tx	Post-Tx	Pre-Tx	Post-Tx	Pre-Tx	Post-Tx	Pre-Tx	Post-Tx	Basal	Stimulated	Basal	Stimulated
	mM		%				*nM/liter*		*μU/ml*			
8	19.6	9.5	7.9	5.6	0.37	0.85	0	0.1	1.6	1.3	3.6	8.8
9	18.2	8.9	6.8	5.2	0.32	0.98	0	0.1	2.3	2.4	4.5	9.5

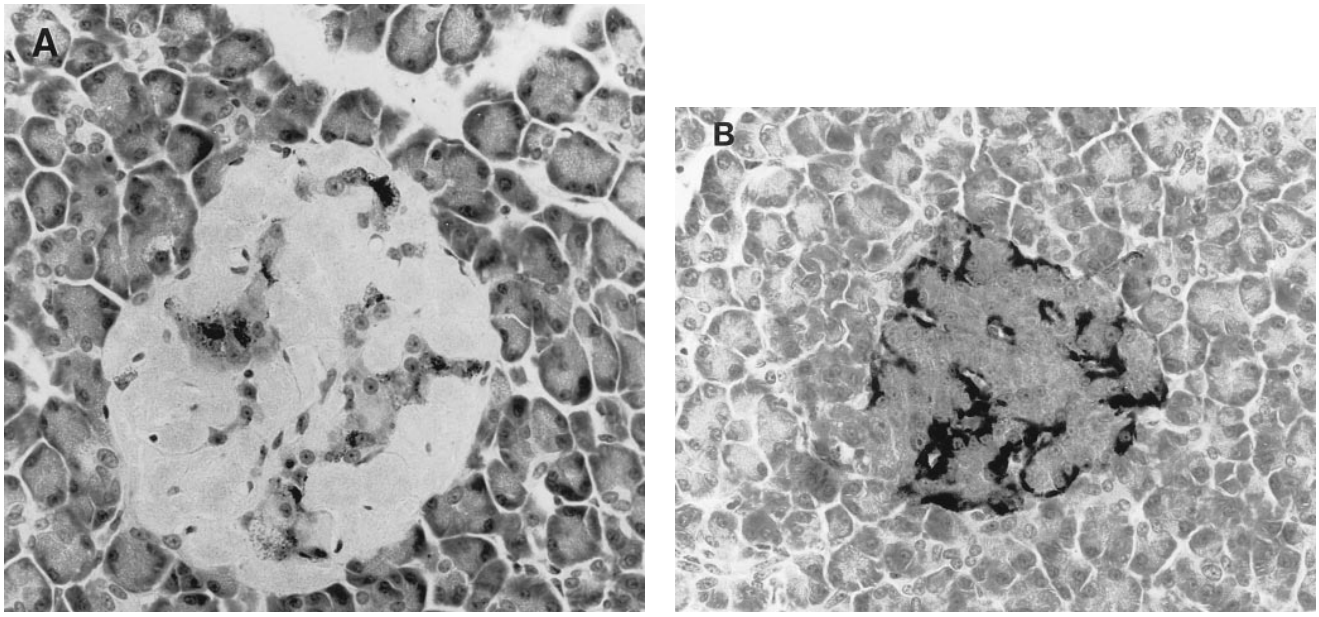

Figure 3. (*A*) Immunostaining for insulin (peroxidase method) of an islet of a naturally diabetic monkey, showing only trace amounts of insulin-positive staining throughout the islet. ×450. (*B*) Immunostaining for glucagon (peroxidase method) of pancreatic islets of a naturally diabetic monkey, indicating the presence of glucagon in α cells on the periphery of the islet. ×400.

spleen appeared normal. No signs of neoplastic changes were detected. The histological evaluation of the aldehyde-fuchsine–stained section of the two animals' pancreata revealed that the islets were depleted of β cells and no insulin was detected in the islets. The immunostaining of the sections showed only trace amounts of insulin within the islets (Fig. 3 *A*), while the glucagon content appeared normal (Fig. 3 *B*).

Discussion

In this study we have for the first time demonstrated that spontaneous diabetes can be reversed for significant periods of time by immunoprotected islet xenografts without recourse to exogenous insulin therapy or to immunosuppression. The long survival of the microencapsulated graft in these experiments can be attributed to two major factors: the strength of the capsular membrane and the purity of the porcine islet tissue. A significant improvement in the strength of the capsules was achieved by the development of the electrostatic droplet generator. The greater strength of the capsules is directly related to their smaller size (0.25–0.35 mm in diameter as opposed to 0.8 mm for capsules generated by the older air-jet technique). The improved capsular strength results in virtually no breaks of the grafted capsules as demonstrated in our recent study (13), thus significantly improving the graft survival. In addition, the smoother surface of the new capsules is conducive to a more efficient coating with both the poly-L-lysine and alginate, thus improving the biocompatibility of the capsules.

The chemical composition of the alginate has been considered by some investigators as crucial in attaining a high degree of biocompatibility of their microcapsules. The fibrosis of implanted microcapsules, experienced by other groups (14–17) was attributed to the mannuronic acid residues acting as cytokine inducers, while another study (18) claimed that a final coating with a high mannuronic acid–content alginate would actually reduce the amount of fibrosis around the microcapsules. However, during the process of the alginate-poly-L-lysine alginate membrane formation, acidic groups of mannuronic and guluronic acids react with the amino groups of the poly-L-lysine and as a result of the proper reaction the resulting capsules should have very little mannuronic or guluronic acid residues exposed. Elimination of either mannuronic or guluronic acid residues from the alginate molecule would result in a considerably weaker capsule. In addition, properly constructed capsules are effective in protecting pancreatic islets against cytotoxic damage by cytokines (19).

The purity of the alginate is much more important for the biocompatibility of the capsules than the alginate composition. This has been also reported by other investigators. Zimmermann et al. (20) have produced a method of preparing mitogen-free alginates. This work has incorporated the development of a mixed lymphocyte response assay to test the purity of the alginates and, as already suggested, has demonstrated that it is the purity and not the chemical composition of the alginate which affects the biocompatibility of the capsules the most.

Clinical transplantation of human islets from cadavers proved that transplantation can normalize hyperglycemia in diabetic recipients (2–5). However, the limited supply of human islets and the difficulties with harvesting pure islets, especially when the procurement of cadaver pancreata is not controlled, make the use of human islets impractical.

The many improvements in our method of porcine islet isolation resulted in islet tissue of 95% purity. This reduction or entire elimination of the contaminating endocrine tissue again positively affected the duration of the graft function. We strongly believe that this improvement in the islet purity along with the improvement in the quality of microcapsules represent the two most important factors affecting the length of the graft survival (11, 13). The use of donor pigs of a well-defined

genetic background contributed further to the resulting reproducibility of the procedure by eliminating the immense variation in the tissue structure of pancreata of randomly selected animals.

The long-term survival of the encapsulated xenograft previously demonstrated in rodents was reconfirmed in this preclinical study in primates. We have shown in a large animal model the ability of the encapsulated graft to achieve physiological glucose-insulin kinetics. Of equal importance is our finding that the until now elusive porcine islets can be isolated with relative ease while retaining their physiological competence and that the immunoisolated porcine xenografts can effectively reverse diabetes in long-term experiments in primates.

This is the first report on long-term discordant xenograft function resulting in a physiological glycemic control without recourse to immunosuppression in a large animal model. The results of this study warrant a clinical trial in human diabetics.

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