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Long-term reversal of diabetes by the injection of immunoprotected islets

(alginate microcapsule/islet allograft/dogs)

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ABSTRACT **The intraperitoneal injection of insulin-producing islets immunoprotected by an alginate–poly(amino acid) membrane is a potential method of reversing diabetes without the need for lifelong immunosuppression. Previous attempts to demonstrate this technology in large animals have failed, preventing application in humans. We have determined that key factors responsible for these past failures include cytokine (interleukins 1 and 6 and tumor necrosis factor) stimulation by mannuronic acid monomers from alginate capsules with weak mechanical integrity, which results in fibroblast proliferation. With this insight, we formulated mechanically stable microcapsules by using alginate high in guluronic acid content and report prolonged reversal of diabetes in the spontaneous diabetic dog model by the intraperitoneal injection of encapsulated canine islet allografts. Euglycemia, independent of any exogenous insulin requirement, was noted for up to 172 days. Graft survival, evidenced by positive C-peptide release, was noted for as long as 726 days in a recipient receiving a single injection of immunoprotected islets. Histological evidence of viable islets retrieved from the peritoneal cavity 6 months posttransplant confirmed the biocompatibility and immunoprotective nature of this capsule formulation. The finding that intraperitoneal injection of alginate-immunoprotected islets, a minimally invasive surgical procedure, is effective in prolonged (>1 year) maintenance of glycemic control, without the need for lifelong immunosuppression, may have significant implications for the future therapy of type I diabetes in humans.**

Diabetes remains the third leading cause of death in the United States. The ability to replace insulin by transplantation of insulin-producing islets in a nonsurgical manner and without the need for lifelong immunosuppression remains a challenge.

Lim and Sun (1) reported reversal of diabetes in rats by microencapsulated islet transplantation over a decade ago. Repeated attempts by several investigators since then have failed to demonstrate reversal of diabetes by this method in large animal models (1–5), thus frustrating the clinical application of encapsulated islets.

For the alginate-based capsule to be successfully applied clinically, it is essential that the microcapsule be biocompatible, mechanically stable, and provide adequate kinetics of insulin secretion in response to a glycemic challenge. The capsule formulations reported to date (1–5), designated type I capsules, fail to meet these criteria. We have identified fundamental factors responsible for this failure (6–8).

There have been no reports on the role of the chemical composition of alginate as it relates to the biocompatibility and mechanical integrity of the capsule. Alginate is a family of polysaccharides composed of homopolymeric regions of β-D-mannuronic (M) and α-L-guluronic (G) acid interspaced with mixed sequences (M–G blocks). The majority of encapsulated islet studies over the past decade report the use of commercial alginate from algae with a high mannuronic acid content (1–5). Until our recent reports (6–8), it had not been recognized that mannuronic acid residues in alginate are potent inducers of cytokine (interleukins 1 and 6 and tumor necrosis factor) release. Since these cytokines are known to be important stimulators of fibroblast proliferation (9, 10), we hypothesized that the fibrosis noted by investigators over the past decade may, in part, be a consequence of the high mannuronic acid content in microcapsule formulations. We demonstrated that microcapsules with high mannuronic acid content retrieved from the peritoneal cavity of Lewis rats revealed intense cellular overgrowth (6), confirmed by EM studies showing activated macrophages and fibroblast proliferation. In contrast, type II microcapsules with high guluronic acid content and increased mechanical stability were largely free of cellular overgrowth as confirmed by histologic and EM examination of retrieved capsules.

Having demonstrated that cytokine stimulation by mannuronic acid was an important component of the fibroblastic response, we hypothesized that a brief, temporary course of an anti-inflammatory agent might prevent this response from any potential mannuronic acid leakage from type II microcapsules. *In vitro* studies confirmed that cyclosporine (CsA), in a dose–response fashion, inhibited mannuronic acid-induced cytokine release (Fig. 1). In a pilot study in a pancreatectomized dog transplanted with an encapsulated islet allograft, we explored the use of a subtherapeutic dose of CsA as an anti-inflammatory agent by instituting a temporary (30 day) course of the drug at doses barely detectable in the blood by HPLC (≤30 ng/ml). In this animal, euglycemia was maintained for 120 days, even after all CsA had been discontinued by day 30. Based on this information, CsA at subtherapeutic dosages were used as an anti-inflammatory agent during the initial proof of principle trials in spontaneous diabetic dogs (11). Further studies in spontaneous diabetic dogs (P.S.-S. and E.F., unpublished data) suggest that a 7-day course of prednisone is as effective as subtherapeutic dosages of CsA in preventing cytokine stimulation in this acute posttransplant period and that with further modifica-

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Abbreviations: CsA, cyclosporine; IVGTT, intravenous glucose tolerance test.

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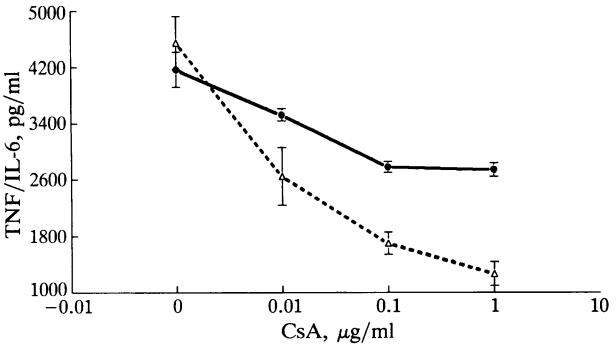

FIG. 1. CsA inhibition of poly(mannuronic acid)-stimulated cytokine release. CsA was added 24 hr prior to the addition of poly(mannuronic acid) at 1 μg/ml, and supernatants were harvested 24 hr later for interleukin 6 (●) and tumor necrosis factor α (△) assays. Spontaneous background production of interleukin 6 (IL-6) and tumor necrosis factor (TNF) were 99 ± 4 and 627 ± 230 pg/ml, respectively, and were unaffected by CsA itself.

tions of type II microcapsules, anti-inflammatory agents may not be necessary.

The aim of this study was to assess the safety and efficacy of multiple retransplants of encapsulated islets in reversing diabetes, as well as to evaluate the ability of these immunoprotected islets to achieve long-term glycemic control.

MATERIALS AND METHODS

To participate in this study, each dog must have had typical features of human type I diabetes mellitus (Table 1), including clinical signs (polyuria, polydipsia, polyphagia, and weight loss), persistent fasting hyperglycemia, persistent glycosuria, and the need for daily injections of insulin to prevent rapid decompensation.

Ten spontaneous diabetic dogs were enrolled. Seven received encapsulated islets and three control dogs received free islets at a dose of 15,000–20,000 islets per kg. Of the seven dogs receiving encapsulated islets in the initial trial (11), five were available for follow-up for 2 years, four of which received retransplantation. Three dogs received a second transplant after loss of insulin independence; at the time of the second transplant, they still had ongoing islet function from the first transplant. One dog received a third injection of encapsulated islets.

Islets were isolated from donor mongrel dogs, and free islets were encapsulated and transplanted as described (11).

In this follow-up study, the anti-inflammatory dose of CsA was discontinued 30 days after transplantation in all five dogs. Thus, to date these recipients have been followed for a range of 110 to >600 days on no immunosuppressive agents.

To assess long-term islet function, fasting blood glucose, plasma C peptide, urine glucose, and ketones were closely monitored after transplantation. In addition, intravenous glucose tolerance tests (IVGTTs) were performed 7 days prior to transplantation in each dog and at regular intervals after transplantation to assess clearance of a systemic glucose challenge.

The following objective criteria were used to confirm ongoing graft survival: (*i*) fasting and/or IVGTT-stimulated serum C-peptide concentration within 2 SD of the normal basal mean (0.22 ± 0.03 pmol/ml)¶, (*ii*) individual glucose clearance rates following intravenous glucose stimulation (K values) greater than 2 SD above the pretreatment mean for these five dogs (0.4 ± 0.19) and within 2 SD of the normal mean (3.96 ± 0.94)¶, (*iii*) absence of diabetic ketoacidosis, (*iv*) serum glycosylated hemoglobin (HbA1c) values less than the pretransplant level, and (*v*) histological evidence of viable islets on retrieval of the capsules from the peritoneal cavity. Positive C-peptide release was considered the most objective criterion of ongoing islet function; thus, the time that elapsed between the first transplant and the last measurement of positive C-peptide release was used to calculate the duration of ongoing islet survival in each dog.

RESULTS

Each dog experienced two phases after transplantation of encapsulated islets. During the first phase, the dogs were clinically normal, independent of any exogenous insulin requirement; they demonstrated fasting euglycemia and absence of glycosuria, with no clinical signs of diabetes mellitus.

The second phase was the period of continued graft function as confirmed by the five criteria described above, following recurrence of hyperglycemia (serum glucose >200 mg/dl) and a need for a minimal dose of supplemental insulin to control clinical signs of diabetes.

Insulin Independence. The duration of insulin independence in each dog is presented in Table 2. In the four dogs receiving a second injection of encapsulated islets, the duration of insulin independence from the first transplant (125 ± 33 days; range, 95–172 days) did not differ significantly from the duration of insulin independence following the second injection (102 ± 20 days; range, 83–130 days). The recipient that received a third injection demonstrated insulin independence for 172, 83, and 138 days following the first, second, and third transplant, respectively.

In contrast, in the three control dogs receiving unencapsulated islets, insulin independence was maintained for a mean of <7 days, with rejection occurring on days 3, 7, and 10.

Long-Term Islet Function. Long-term islet function was demonstrated in each of the five recipients receiving encapsulated islets. Table 2 summarizes the natural history of immunoprotected beta-cell function following intraperitoneal injection in each of the five recipients.

Duration of Graft Survival. Since the primary objective of the study was to determine the safety and efficacy of multiple retransplants, four recipients received a second transplant

¶Normal K values and normal fasting C-peptide values were obtained by performing IVGTTs in 21 normal dogs.

Table 1. Pretransplant entrance criteria

Criteria	Requirement(s)
Severe diabetic ketoacidosis*	One occurrence within 6 months pretransplant
IVGTT glucose clearance rate (K value)	K value >3 SD below normal mean†
Fasting basal and stimulated (IVGTT) plasma C peptide	Fasting basal and stimulated C peptide >3 SD below normal basal mean†
Absolute insulin deficiency	Daily insulin therapy

*Severe ketoacidosis is defined as clinical signs of vomiting and anorexia, plus ≥5% dehydration, total venous CO_2 >12 milliequivalents per liter, blood glucose >300 mg/dl, ketouria, and requiring at least 48 hr of intensive care.
†The normal mean was obtained from analysis of K values (normal mean = 3.9 ± 0.9) and C-peptide levels (normal mean = 0.22 ± 0.03 pmol/ml) from 21 normal dogs.

Table 2. Duration of insulin independence and long-term graft survival

Dog	Insulin independence		Graft survival,*	K value		Fasting C peptide, pmol/ml		Stimulated C peptide, pmol/ml		HbA1c, g/dl	
no.	Tx no.	Days	days	Pre-Tx†	Last point‡	Pre-Tx	Last point‡	Pre-Tx	Last point‡	Pre-Tx	Last point‡
1	1	172									
	2	83	641	0.44	1.61 (734)	0.11	0.32 (641)	0.11	0.49 (605)	9.6	2.8 (676)
	3	138									
2	1	95	228§	0.69	1.40 (620)	0.11	0.25 (228)	0.12	0.20 (620)	8.9	7.7 (234)
	2	95									
3	1	115	550	0.18	0.90 (550)	0.07	0.18 (550)	0.07	0.26 (550)	7.8	6.1 (496)
	2	98									
4	1	119	269¶	0.35	1.50 (269)	0.05	0.24 (269)	0.07	0.29 (269)	8.8	4.7 (269)
	2	130									
5	1	56	726	0.46	0.85 (599)	0.08	0.18 (726)	0.10	0.20 (599)	9.3	6.0 (717)
Mean ± SD‖		110 ± 32	483 ± 223	0.42 ± 0.19	1.25 ± 0.35**	0.08 ± 0.03	0.23 ± 0.06**	0.09 ± 0.02	0.29 ± 0.12**	8.9 ± 0.68	5.5 ± 1.8**

Tx, treatment.
*The duration of graft survival was calculated from the last day of measurement of positive C-peptide release.
†Entrance data only (single observation data at day 3 pretreatment).
‡The value in parentheses is the day of the last measurement.
§Euthanized on day 620 with ongoing islet function.
¶Euthanized on day 269 with ongoing islet function.
‖$n = 5$ except for the number of days of insulin independence, for which $n = 10$.
**Significantly different from the pretreatment value at $P < 0.05$.

upon loss of insulin independence but with ongoing islet function from the first transplant. Thus, in these four recipients, it is difficult to determine the maximum duration of graft function possible from a single dose of encapsulated islets, since the second transplant was performed on days 172, 95, 115, and 119 respectively, with ongoing islet function from the first transplant. Long-term graft survival after multiple transplants in these four recipients was 641, >228, 550, and >269 days (Table 2).

Duration of Graft Survival After a Single Injection. Insight into the potential duration of long-term graft survival after a single injection is provided by recipient no. 5 (Tables 2 and 3). To date this recipient has not been retransplanted and has demonstrated continuous islet function for >726 days from a single encapsulated islet transplant as evidenced by ongoing basal C-peptide secretion (0.18 pmol/ml) on that date. Analysis of C-peptide secretion and glycemic control over the course of the natural history of the transplant in this recipient (Table 3) is instructive regarding the long-term function of a single injection of immunoprotected cells. Euglycemia (serum glucose = 115 mg/dl), independent of any insulin injections, was achieved posttransplant with a K value of 4.0 and basal C-peptide secretion of 0.76 pmol/ml. C-peptide secretion gradually decreased over time from 0.76 pmol/ml to 0.40 pmol/ml, after which insulin supplementation was required. Ongoing islet function, as evidenced by C-peptide secretion within 2 SD of the normal basal mean was noted throughout the course of this period of insulin supplementation. At the time of this report, this recipient has ongoing graft survival, 726 days since the transplant. Despite a 50% reduction in insulin dose (ultralente insulin given daily), glycemic control was significantly improved compared to pretransplant levels, as evidenced by improved glucose clearance rates (K values), HbA1c of 6.0 (day 717), and absence of a single episode of ketoacidosis to date. Ongoing islet function has continued in this recipient for >600 days without any immunosuppression, providing evidence for the immunoprotective nature of this type II microcapsule.

Long-term Glycemic Control. Pretransplant basal and glucose-stimulated C-peptide release, K values, glycosylated hemoglobin levels, and the number of episodes of ketoacidosis were determined for each of the five dogs receiving encapsulated islets (Table 4). Multiple determinations were obtained for each variable throughout the natural history of the transplant in each dog. Basal C-peptide levels, peak C-peptide release, K values, HbA1c, and episodes of ketoacidosis during periods of insulin independence and during periods of insulin supplementation following the first, second, and third transplant were compared to multiple observations taken during the pretransplant period. The data was analyzed using t tests at a significance level of $P < 0.05$. Long-term islet function and tight glycemic control were

Table 3. Graft survival (C-peptide release) and glycemic control after a single injection of encapsulated islets

Time	Fasting serum glucose, mg/dl	Insulin dose, units/24 hr	Fasting C peptide, pmol/ml	Stimulated C peptide, pmol/ml	K value	HbA1c	Ketoacidosis, no. of episodes
Pretreatment	280	20–22	0.08	0.10	0.46	9.3	1–2/yr
Posttreatment							
Day 14	115	0	0.76	0.93	4.00	7.2	0
Day 28	85	0	—	—	—	5.6	0
Day 46	106	0	0.48	—	—	—	0
Day 60	250	0	0.40	0.38	—	—	0
Day 63*	—	10	—	—	—	—	0
Day 461	208	10	0.18	0.25	1.50	6.8	0
Day 599	221	10	0.13	0.20	0.85	—	0
Day 717	—	10	—	—	—	6.0	0
Day 726	234	14	0.18	—	—	—	0

*Insulin supplementation started on day 63 (10 units of ultralente per 24 hr).

Table 4. Long-term glycemic control

Measurement	Pretreatment		Insulin independence		Insulin supplementation	
	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD	*n*
Blood glucose, mg/dl	278 ± 120	41	106 ± 35*	196	225 ± 93*	34
K value	0.77 ± 0.50	9	2.18 ± 0.90*	22	1.18 ± 0.43*	14
Fasting C peptide, pmol/ml	0.13 ± 0.07	13	0.57 ± 0.48*	171	0.18 ± 0.07*	13
Stimulated C peptide, pmol/ml	0.15 ± 0.08	8	0.57 ± 0.26*	15	0.26 ± 0.08*	8
HbA1c, g/dl	7.81 ± 1.6	10	5.02 ± 1.3*	37	6.68 ± 2.01	12

n, Number of observations taken throughout the natural history of all five recipients.
*Significantly different from the pretreatment value at $P < 0.05$.

noted in all five recipients as evidenced by significant improvements in HbA1c levels, K values, and C-peptide release (Table 4).

Diabetic Ketoacidosis. Absence of even a single episode of diabetic ketoacidosis in each of the five recipients after transplantation provides significant evidence of the improvement of glycemic control noted in these animals. Even when modest insulin supplementation was required, ketoacidosis has not occurred at the time of this report in any of the five recipients. This is in contrast to the one or more episodes of ketoacidosis reported in these recipients, prior to transplantation, when on full doses of insulin.

Histological Examination. Histological corroboration of ongoing islet survival is provided by positive evidence of viable islets (positive anti-insulin, anti-glucagon, and anti-somatostatin staining) from omental biopsy specimens retrieved at the time of the second transplant from recipient nos. 1, 2, and 4. Fig. 2 demonstrates encapsulated islets from recipient nos. 1 and 4 retrieved 180 and 139 days, respectively, after injection into the peritoneal cavity. Multiple capsules with intact viable islets were noted, demonstrating the biocompatibility, mechanical integrity, and immunoprotectivity of the capsule membrane.

DISCUSSION

Major complications of diabetes persist despite insulin therapy. The challenge physicians face is to intervene before these secondary complications take their toll. A potential method of meeting this challenge is the transplantation of immunoprotected insulin-producing cells, without the need for lifelong antirejection therapy.

Absence of progress in alginate-encapsulation technology has been largely due to lack of understanding of the fundamental issues relating to fibrosis associated with these capsules. We recently recognized the importance of mannuronic acid in the pathogenesis of this fibrosis (6, 7). The molecular basis of this cytokine stimulatory effect of poly(mannuronic acid) has been partly elucidated, and it has been shown that CD14 on the monocyte membrane is involved in both binding and biological activity of mannuronic acid residues (12).

On the basis of these findings, we devised a type II capsule to overcome unwanted immune responses *in vivo* by the use

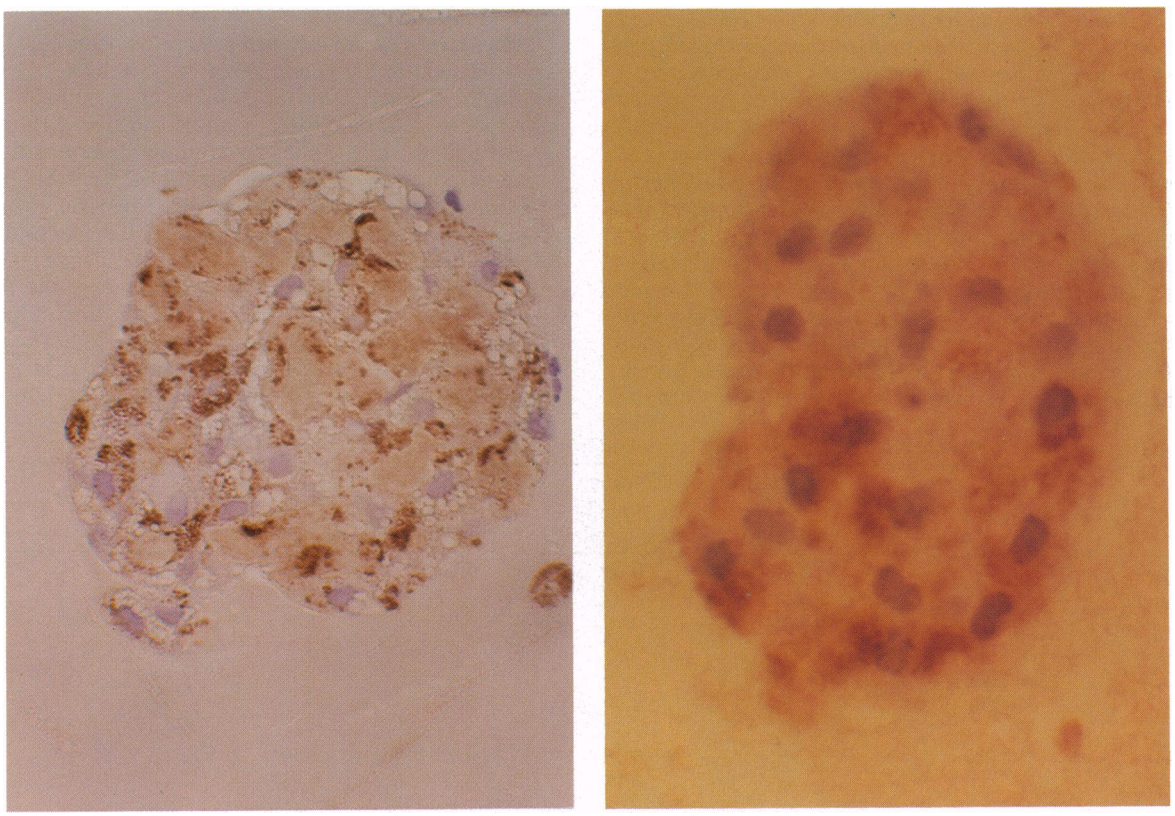

FIG. 2. Encapsulated canine islets retrieved 180 days (*Left*) and 139 days (*Right*) from the peritoneal cavities of recipient nos. 1 and 4, respectively. The positive anti-insulin staining demonstrates ongoing islet viability. (*Left*, ×425; *Right*, ×500.)

of alginate with a high guluronic acid content and by cytokine inhibition using a temporary course of anti-inflammatory pharmacologic agents. Preliminary studies suggest that with improved microcapsule formulations, these anti-inflammatory agents may ultimately not be necessary in this acute posttransplant phase. Further studies are needed to confirm this. Evidence of viable islets retrieved from the peritoneal cavity 180 days after transplantation confirms the immunoprotectivity afforded these islets by the microcapsule membrane and provides evidence of the biocompatibility of this capsule formulation.

To our knowledge, this is the first report of long-term islet function (>1 year) following an intraperitoneal injection of encapsulated islets in the large animal model. Graft survival was 641, >228, 550, and >269 days in the dogs receiving multiple implants and for as long as 726 days in the recipient receiving a single transplant. We chose a strict definition of positive C-peptide secretion as evidence of ongoing graft survival, even though this unfavorably skews our data when compared to the definition of graft survival reported by others in the literature who used reduction of insulin dose as the measure for ongoing islet function. By the latter criteria, ongoing islet survival would be equal to or greater than 780, 619, 720, 269, and 760 days, respectively, in these five dogs.

The extravascular placement of encapsulated islets free-floating in the peritoneal cavity provides a safe, minimally invasive method for reversing diabetes. Insulin release from these nonvascularized islets in response to a systemic glycemic signal must be shown to be satisfactory for clinical applicability. We reported the ability of free-floating intraperitoneally implanted encapsulated rat islets (13) as well as highly discordant encapsulated human islets (14) to normalize a systemic glucose challenge in diabetic Lewis rats. In this report, we demonstrate that in the large animal canine model, intraperitoneal islets encapsulated within this modified type II microcapsule provide a nonvascularized bioartificial organ with the ability to achieve rapid glucose–insulin kinetics. Each recipient in the study achieved rapid normalization of serum glucose in response to an intravenous glucose stimulus, providing evidence for an adequate *in vivo* insulin response from intraperitoneal, free-floating encapsulated islets to a glycemic signal.

Studies in whole organ pancreas transplant recipients suggest that nephropathy may be prevented from recurring in renal allografts (15) and that neuropathy may also stabilize (8). This is significant as it relates to the potential of encapsulated islets to prevent secondary complications of diabetes by achieving good metabolic control. The findings of significant improvements in HbA1c levels, K values, and ongoing C-peptide secretion, with absence of diabetic ketoacidosis in all five recipients, demonstrate the efficacy of intraperitoneal encapsulated islets in achieving long-term glycemic control. In the case of the recipient receiving a single transplant, long-term glycemic control has been achieved for almost 2 years since the intraperitoneal injection of encapsulated islets.

In summary, we describe an alginate-based microcapsule that provides sufficient immunoprotection and diffusion properties to accomplish prolonged glucose homeostasis in the large animal model. This demonstration may have important implications for the future therapy of type I diabetes in humans. If tight homeostatic control of glucose metabolism can be achieved by the minimally invasive technique of an intraperitoneal injection of nonvascularized encapsulated islets, without the need for life-long immunosuppression therapy, the goal of preventing the debilitating secondary complications of type I diabetes may be realized.

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