

# Xenotransplantation of canine, bovine, and porcine islets in diabetic rats without immunosuppression

(diabetes/artificial pancreas/immunoisolation)

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**ABSTRACT** Permeable acrylic membranes were employed to prevent immune rejection of discordant islet xenografts isolated from various large animals. Canine, porcine, and bovine islets were seeded into tubular diffusion chambers and transplanted into the peritoneum of 27 nonimmunosuppressed streptozotocin-induced diabetic Lewis rats. Six recipients received islet grafts from bovine calves, 7 received grafts from pigs, and 14 received grafts from dogs. Four of the latter were removed at 1 month. In the control group of 10 diabetic rats, 4 received nonencapsulated canine islets, 3 received nonencapsulated bovine islets, and 3 received nonencapsulated porcine islets. Recipients of encapsulated islets promptly dropped from a pretransplantation plasma glucose level of  $487 \pm 36$  (mean  $\pm$  SEM) to  $84 \pm 2$  (canine),  $81 \pm 4$  (bovine), and  $81 \pm 3$  mg/dl (porcine) during the first week. All of the animals sustained these levels for at least 1 month. One rat spontaneously reverted to diabetes at 54 days posttransplantation; 4 other rats became hyperglycemic (glucose,  $>600$  mg/dl) after membrane removal on day 30. The remaining 22 rats maintained fasting euglycemia for  $>10$  weeks. In contrast, rats that received nonencapsulated islets became hyperglycemic in  $<7$  days. Intravenous glucose tolerance test K values (decline in glucose levels, %/min) at 1 month for the canine and bovine encapsulated islet transplant group were  $3.5 \pm 0.3$  and  $3.3 \pm 0.1$  compared with  $3.3 \pm 0.1$  ( $P = 0.63$ ) and  $0.91 \pm 0.1$  ( $P < 0.0001$ ) for normal ( $n = 4$ ) and diabetic ( $n = 4$ ) control groups. Morphologic studies of long-term functioning grafts (30–130 days) revealed well-preserved  $\alpha$ ,  $\beta$ , and  $\delta$  cells, with varying degrees of granulation. These results demonstrate that immune isolation of islet tissue using permeable artificial membranes can protect discordant islet xenografts from immune rejection in the absence of any immunosuppressive drugs.

Diabetes afflicts an estimated 80 million people worldwide (1). Even with improved procurement of human organs, the supply of donor tissue would remain quite inadequate if pancreatic islet transplantation were to be developed as an effective therapy. One potential solution to this problem is the use of non-human donor islets. However, the transplantation of xenogeneic tissues of widely divergent species is difficult due to vigorous humoral and cellular immune responses. The most encouraging experience involves the encapsulation of donor islets in artificial membranes that are impermeable to immunocytes and immunoglobulins but that permit crossover of lower molecular weight substances such as glucose and insulin (2, 3). Preliminary studies in rodents have demonstrated that xenografted encapsulated islets can function for up to 210 days (4). Although these experiments have proven successful for

closely related islet xenografts (concordant, rodent-to-rodent combinations), islet xenografts between discordant species add another dimension to the rejection response, in that most discordant species have natural cytotoxic antibodies against each other (5). The development of successful islet isolation techniques for animals such as the cow (6) and pig (7) could enable the use of readily available supplies of large-animal donor tissue for islet grafting into human diabetics. Unfortunately, there is currently no clinically applicable immunologic method available that prevents the destruction of discordant islet xenografts (8–11).

Encouraged by the results obtained with the encapsulation of allogeneic and xenogeneic rodent islets in capillary hollow fibers (4, 12–14), we sought to determine whether larger tubular membrane diffusion chambers (fabricated from XM-50 acrylic copolymer) can protect xenografts of canine, bovine, and porcine islets from rejection in the absence of any immunosuppressive drugs. Because the formation of fibrous tissue around the membrane has been a cause of failure in the past, a smooth external skin was used to minimize this host reaction.

## MATERIALS AND METHODS

**Animals.** Adult male Lewis rats (Charles River Breeding Laboratories) weighing 250–300 g were used as transplant recipients. Animals were fed ad libitum with a standard pelleted diet (Agway, no. 3000 RHM-Prolab) and allowed free access to water. Diabetes was induced by a single injection of streptozotocin (42 mg/kg of body weight in 0.01 M citrate buffer, pH 4.5; Sigma) into the tail vein 10–14 days prior to surgery. Only rats with plasma glucose levels  $>400$  mg/dl were used in these studies. Fasting plasma glucose concentrations were measured by tail bleedings using the glucose oxidase method (Beckman glucose analyzer 2; Fullerton, CA); determinations were performed three times weekly for 30 days and then weekly for the duration of each study. The animals were not fasted the day of or the day after transplant surgery. Failure was considered to have occurred when plasma glucose concentrations exceeded 200 mg/dl on two consecutive determinations.

**Islet Isolation and Encapsulation.** Islets were prepared from either adult mongrel dogs, pigs, or bovine calves (0–2 weeks old) by a modification of the method of Warnock and Rajotte (15). Pancreatic tissue was dissociated using a collagenase digestion procedure, and the islets were separated from

Abbreviation: IVGTT, intravenous glucose tolerance test.

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exocrine tissue on a discontinuous Ficoll density gradient. Isolated islets were then cultured for 1 day either in M199/Earle's medium supplemented with 10% (vol/vol) fetal bovine serum, 20 mM Hepes, 100 mg of glucose per dl, and 400 international units of penicillin per ml (canine) or in  $\alpha$  minimum essential medium plus 10% heat-inactivated horse serum (bovine and porcine) in a humidified atmosphere of 5% CO<sub>2</sub>/95% air at 37°C. After preculturing, the islets were placed in semipermeable tubular membranes (2–3 cm long, 1.77 mm i.d., 69- $\mu$ m wall thickness) with a nominal molecular weight cutoff of 50,000–80,000 (W. R. Grace & Co.). Islets were suspended in 1.2% (wt/vol) Pronova LVG sodium alginate (Protan, Drammen, Norway) at a concentration of 4–6 islet equivalents<sup>||</sup> per mm<sup>3</sup> (200–400 islets per membrane). The cells were seeded into the tubular membrane chambers using a 16-gauge angiocatheter. After gelation in a solution of 1.5% CaCl<sub>2</sub>, the chambers were washed in culture medium, and the ends were sealed by heat followed by dipping in a solution of acrylic copolymer (XM casting solution; W. R. Grace & Co.) similar to that used to fabricate the tubular membranes.

**In Vitro Glucose–Insulin Kinetics.** *In vitro* perfusion was carried out to test the insulin secretory activity from the islets seeded within the capsules and to evaluate the kinetic performance of this type of diffusion-based biohybrid artificial pancreas as an insulin delivery device. Encapsulated and nonencapsulated islets (381  $\pm$  48 equivalents) were perfused with glucose concentrations of 100, 300, and 100 mg/dl for 60 min at each concentration as described (16). The flow rate was 0.5 ml/min and the perfusate was collected with a microfraction collector (Gilson model 203). The samples were frozen for subsequent insulin assay using a standard equilibrium radioimmunoassay protocol as previously described (17). The limit of assay sensitivity was 25 microunits (1 ng)/ml and data reduction was done by a log–logit transformation of the standard curve by COBRA  $\gamma$  counter (Packard Instruments).

**Experimental Design.** Recipient rats were anesthetized with ether inhalation. Free or macroencapsulated islets (1–2  $\times$  10<sup>4</sup> islet equivalents) were introduced into the peritoneal cavity through a small (1–2 cm) midline incision. The wound was closed in two layers with 4-0 silk suture. In the experimental group of 27 rats, 6 received islet grafts from bovine calves, 7 received grafts from pigs, and the remaining 14 received grafts from dogs. Four of the latter were removed at 1 month. In the control group of 10 diabetic rats implanted with nonencapsulated islets, 4 received canine islets, 3 received bovine islets, and the remaining 3 received porcine islets. Intravenous glucose tolerance tests (IVGTTs) were performed at 1 month after transplantation and compared with normal ( $n = 4$ ) and untreated diabetic ( $n = 4$ ) rats. Fifty percent (wt/vol) glucose (0.5 g/kg of body weight) was injected into the tail vein, and plasma glucose concentrations were measured at 0, 1, 5, 10, 20, 30, 40, 50, and 60 min after the glucose injection. K values (decline in glucose levels, %/min) were calculated according to standard methods (18).

**Histology.** The islet implants and the pancreas of recipient animals were fixed in Bouin's solution, dehydrated, and embedded in paraffin. Five-micron sections were prepared and stained with hematoxylin/eosin and for the presence of insulin, glucagon, and somatostatin using immunoperoxidase techniques (19). Two representative longitudinal sections of each native pancreas were examined.

**Statistical Analysis.** Data are presented as mean ( $\bar{x}$ )  $\pm$  SEM and compared by using the unpaired Student's *t* test or one-way analysis of variance (ANOVA). In conjunction with

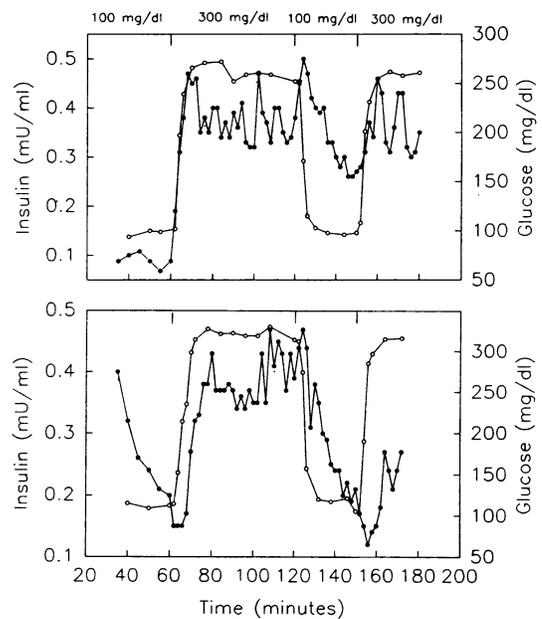


FIG. 1. Insulin (●) secretory response to glucose (○) by perfused nonencapsulated (Upper) and macroencapsulated (Lower) canine islets. The increase in insulin release from encapsulated islets was only slightly delayed in comparison to nonencapsulated islets. mU, milliunits.

ANOVA, multiple comparison methods of either Tukey or Scheffe were used, with a significance level of 0.05. Correlation was analyzed by univariate linear regression with the STATVIEW II program (Abacus Concepts, Berkeley, CA).

**RESULTS**

**In Vitro Glucose–Insulin Kinetics.** The *in vitro* insulin secretory response of macroencapsulated and nonencapsulated islets is shown in Fig. 1. An  $\approx$ 4-fold average increase from the basal insulin secretion was observed in both groups. The secretory response of the macroencapsulated islets was sustained for 60 min of glucose stimulation (300 mg/dl) and returned to basal levels after perfusion with the low-glucose solution (100 mg/dl). Five separate experiments in which this protocol was used indicated a delay of 7  $\pm$  1 min before insulin concentration in the perfusate began to increase. This finding indicates that the islets encapsulated by this procedure were functionally intact and could respond rapidly to an increase in glucose concentration.

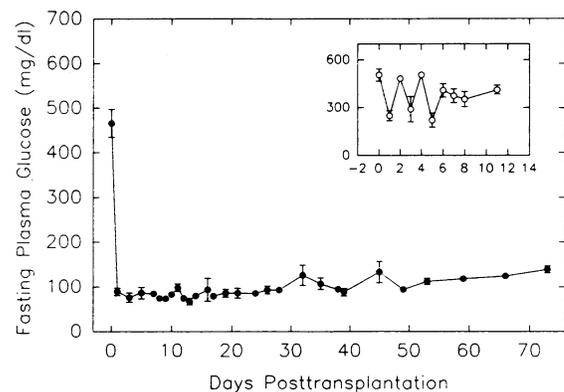


FIG. 2. Fasting plasma glucose levels ( $\bar{x} \pm$  SEM) in 10 diabetic rats that received intraperitoneal implants of macroencapsulated canine islets. One graft failed at 54 days posttransplantation. (Inset) Nonencapsulated canine islets ( $n = 4$ ).

<sup>||</sup>A unit introduced to standardize islet number, equivalent to the volume of an average islet of 150  $\mu$ m in diameter.

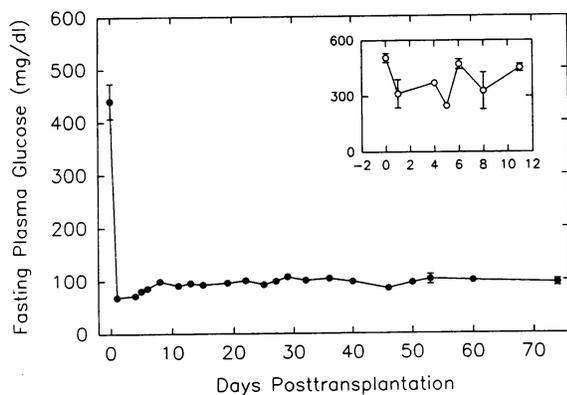


FIG. 3. Fasting plasma glucose levels ( $\bar{x} \pm \text{SEM}$ ) in six diabetic rats that received intraperitoneal implants of macroencapsulated bovine islets. (Inset) Nonencapsulated bovine islets ( $n = 3$ ).

**Transplantation.** Intraperitoneal transplants of  $1\text{--}2 \times 10^4$  encapsulated islets reversed the diabetic state of all 27 recipient rats within 24 hr (Figs. 2–4). Plasma glucose levels ( $\bar{x} \pm \text{SEM}$ ) dropped from a pretransplantation level of  $487 \pm 36$  to  $84 \pm 2$  (canine),  $81 \pm 4$  (bovine), and  $81 \pm 3$  mg/dl (porcine) during the first week. All of the animals sustained these levels for at least 1 month. One rat subsequently reverted to diabetes at 54 days posttransplantation and four canine islet xenograft recipients reverted to diabetes after membrane removal on day 30. The remaining 22 rats maintained fasting euglycemia for >10 weeks. In contrast, non-encapsulated islet transplants only partially reversed the diabetic state of the recipients, with hyperglycemia returning to pretransplantation levels after 6–7 postoperative days (Figs. 2–4 Insets).

**Metabolic Studies.** The results of IVGTTs on normal control and transplanted rats are shown in Table 1. Glucose clearance (K) rates of the animals transplanted with encapsulated islets were not statistically different from those of normal rats after the administration of glucose ( $P = 0.63$ ). However, the IVGTT profiles were not totally normal, although plasma glucose declined promptly, reaching near-normal levels after 60 min and resulting in K values of  $3.5 \pm 0.3$  (canine islets),  $3.3 \pm 0.1$  (bovine islets), and  $3.3 \pm 0.1$  (normal). By contrast, the untreated streptozotocin diabetic rats remained significantly hyperglycemic after glucose challenge, with K values impaired at  $0.91 \pm 0.1$  ( $P < 0.0001$ ).

**Histology and Membrane Removal.** To check for evidence against spontaneous recovery by  $\beta$  cells, pancreata from the experimental and control groups were routinely fixed and processed. The histological appearance is shown in Fig. 5 for

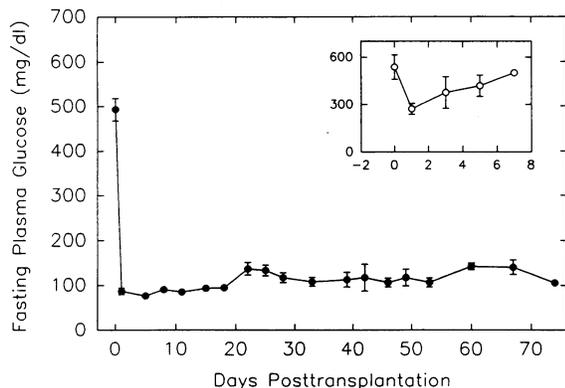


FIG. 4. Fasting plasma glucose levels ( $\bar{x} \pm \text{SEM}$ ) in seven diabetic rats that received intraperitoneal implants of macroencapsulated porcine islets. (Inset) Nonencapsulated porcine islets ( $n = 3$ ).

Table 1. Fasting plasma glucose concentrations ( $\bar{x} \pm \text{SEM}$ ) and IVGTT K values at 1 month in transplanted, normal, and untreated diabetic rats

Parameter	Transplanted islets		Normal	Diabetic
	Canine	Bovine		
Plasma glucose, mg/dl	$82 \pm 7$	$101 \pm 1$	$94 \pm 1$	$347 \pm 32$
K value, %/min	$3.5 \pm 0.3$	$3.3 \pm 0.1$	$3.3 \pm 0.1$	$0.91 \pm 0.1$
Number of animals	4	3	4	4

the pancreas recovered from a normal control animal (Fig. 5A) and a streptozotocin-treated animal sacrificed 93 days after transplantation with encapsulated canine islets (Fig. 5B), respectively. The latter pancreas exhibited signs of streptozotocin toxicity with loss of normal islet architecture. Virtually no granulated  $\beta$  cells were seen. Histology of the membranes retrieved from this animal showed well-preserved islets, interspersed with solid, basophilic alginates, much as in freshly seeded chambers. Overall, perhaps 75–80% of the seeded endocrine tissue remained viable. Cells staining for glucagon and somatostatin were also found among the  $\beta$  cells in most section planes.

To confirm further that the euglycemic state of the transplant recipients resulted from the transplantation of macroencapsulated islets, membranes containing encapsulated canine islets were removed 30 days after implantation in four rats. Hyperglycemia was subsequently observed in all animals, with plasma glucose levels rising to >600 mg/dl within 3 days (Fig. 6). The implanted membranes were generally free floating and free of cellular overgrowth. The external surfaces were free of fibrosis and host cell adherence. On microscopic examination, the membranes contained up to 85–90% viable islets. Immunohistochemical staining of the islets revealed hormone-producing cells, with varying degrees of  $\alpha$ ,  $\beta$ , and  $\delta$  granulation (Fig. 7).

## DISCUSSION

Transplantation of islets across a wide species barrier has been difficult to achieve. Islets isolated from large mammals

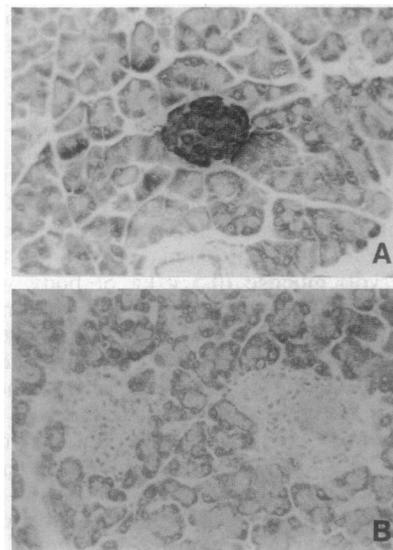


FIG. 5. Immunoperoxidase stain for insulin of the pancreas recovered from normal control rat (A) and streptozotocin-treated animal sacrificed 93 days after transplantation with encapsulated canine islets (B). ( $\times 230$ .) The latter pancreas exhibits signs of streptozotocin toxicity, with loss of normal islet architecture. Virtually no granulated  $\beta$  cells are seen. The background stained with hematoxylin.

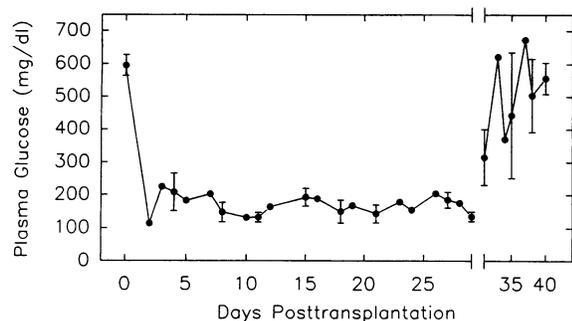


FIG. 6. Random plasma glucose levels ( $\bar{x} \pm \text{SEM}$ ) in four diabetic rats that received intraperitoneal implants of macroencapsulated canine islets. The membranes were removed at 1 month.

and humans and transplanted into nonimmunosuppressed diabetic rats are rapidly rejected (20, 21). In the present study, prolongation of discordant xenograft survival has been achieved in diabetic rats using artificial permselective membranes. Tubular membranes containing canine, bovine, or porcine islets routinely normalized fasting plasma glucose levels after transplantation into streptozotocin-induced diabetic rats. All of the grafts functioned for 1 month, and for at least 10 weeks in bovine and porcine islet xenograft recipients.\*\* In comparison, nonencapsulated islet transplants sustained euglycemia for <1 week.

Although prolongation of survival of islets from discordant species has been achieved by microencapsulation, these studies have been performed mainly in mice and have usually required adjunctive treatment with immunosuppressive agents

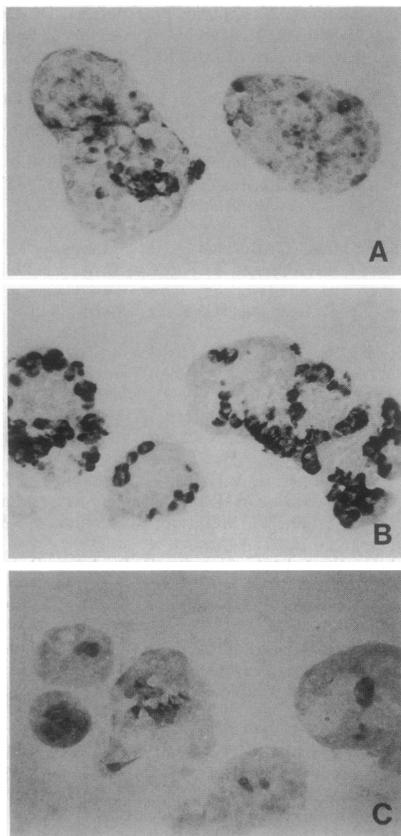


FIG. 7. Encapsulated canine islets retrieved from the peritoneal cavity of diabetic rats 30 days after xenotransplantation. Immunoperoxidase stained for insulin (A), glucagon (B), and somatostatin (C), revealing multiple, viable, hormone-containing endocrine cells. ( $\times 570$ .)

(22–24). Weber *et al.* (8) found that alginate/poly(lysine) microcapsules containing  $4 \times 10^3$  to  $1.2 \times 10^4$  canine islets<sup>††</sup> functioned for only  $11.5 \pm 3$  days (range, 5–17 days) in nonobese diabetic mice. With anti-CD4 monoclonal antibody treatment, however, long-term functional survival was observed for 4/8 (50%) recipients. Histologic examination of the microcapsules at the time of rejection revealed the presence of macrophages, multinucleate giant cells, granulocytes, and lymphocytes, consistent with an inflammatory reaction. It is likely that, once recruited to the surroundings of the microcapsule, lymphokine-secreting cells injured the xenogeneic islets via soluble factors. This is in keeping with reports that the microcapsule membrane is permeable to the immunomodulatory polypeptide products of lymphocytes, macrophages/monocytes, and natural killer cells of the immune system (2, 25) and that a number of cytokines (interferon  $\gamma$  and interleukin 2) and monokines (interleukin 1 and tumor necrosis factor) induce the degeneration of islet cells (26–28).

Recently, Zekorn *et al.* (29) have shown that rat islets are protected from interleukin 1 toxicity by hollow fiber membranes. A high dose of recombinant human interleukin 1 $\beta$  resulted in an inhibition of the glucose-stimulated insulin release of free islets, whereas encapsulated islets showed a preserved insulin release. Therefore, the use of hollow fiber and tubular membrane devices might have potential advantages, especially when engraftment is carried out across a wide species barrier. Indeed, Altman *et al.* (30) reported that intraperitoneal transplants of human islets and human insulinoma tissue into nonobese diabetic mice had a survival time of <2 weeks, and the survival was increased to >6 months (two-thirds of the animals in each group) when encapsulated in hollow fiber membranes. However, this type of fiber was observed to elicit an inflammatory pericapsular response in the rat (30, 31) and the large-animal (porcine) model (32). The fibrotic reaction in the rats was qualitatively similar to that seen in the pig, except for lymphoid clusters with giant and pseudo-epithelioid cells that were observed only in pigs. The reaction consisted of a layer (<50  $\mu\text{m}$ ) of fibroblasts and collagen with polymorphonuclear leucocytes, macrophages, histiocytes, and small lymphocytes. The fiber wall was always infiltrated by collagen, fibroblasts, and macrophages.

Experiments performed in our laboratory corroborate these findings. We compared the use of tubular diffusion chambers with a smooth skin versus a rough skin—i.e., an open trabecular or honeycombed surface—on the outside surface. Membranes with rough external surfaces elicited a significant fibrotic response in streptozotocin-induced diabetic rats in <1 week. In contrast, smooth membranes retrieved >10 weeks after implantation showed no evidence of an inflammatory response. The external membrane surfaces were generally free of fibrotic encapsulation and exhibited only occasional host-cell adherence (Fig. 8). The response was not influenced by seeding or nonseeding of the chambers or by the type of tissue inside (canine, bovine, or porcine islets). Protection of the xenogeneic tissue by the membrane is demonstrated by the preservation of the structure of the islets residing for >6 months in the peritoneal cavity (Fig. 9) as well as by the presence of hormone-producing cells containing varying degrees of  $\alpha$ ,  $\beta$ , and  $\delta$  granulation. Occasionally the chambers were broken or ex-

\*\*Most of the animals (9/16) have continued to maintain fasting euglycemia, with functional survival now >200 days.

††Although hyperglycemia in rodents has been reversed by interportal or subrenal capsule transplantation using fewer islets, these studies have been performed mainly with free, nonencapsulated islets. Dose-response curves performed in our laboratory using encapsulated islets indicate that larger numbers of islets are necessary to achieve long-term normoglycemia in the large-animal-to-rodent encapsulated islet xenograft model.

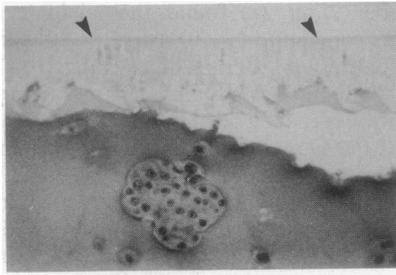


FIG. 8. Encapsulated porcine islets retrieved from the peritoneal cavity of a diabetic rat 82 days after xenotransplantation. (Hematoxylin/eosin;  $\times 570$ .) Note the absence of fibrosis and host cell adherence on the external surface (arrowheads).

hibited bends that resulted in fracture of the external and internal membrane walls. Such complete fractures permitted xenograft rejection within the interior characterized by the presence of fibroproliferative connective tissue and immune cell elements. Islet cell survival within the alginate matrix was markedly reduced within these damaged membranes.

Although our results with survival of islet xenografts in streptozotocin-induced rats are encouraging, it will be important to utilize this encapsulation technology in spontaneously diabetic animals such as BB rats or nonobese diabetic mice in which diabetes is believed to result from autoimmune  $\beta$ -cell damage. Preliminary experiments in diabetic BB/Wor rats suggest that canine islet implants can restore euglycemia for  $>150$  days without immunosuppression. In addition, this should eliminate the possibility of  $\beta$ -cell regeneration that has been reported for the streptozotocin model of diabetes (33). In related studies in our laboratory we have also observed that removal of islet implants after periods of several weeks did not always result in subsequent large rises in plasma glucose concentrations.

Our results demonstrate the feasibility of long-term immunosolation of discordant islet xenografts by artificial membranes and the long-term biocompatibility of the membrane versus the graft and versus the recipient. However, to function as an implantable biohybrid artificial pancreas, it is necessary that the islets placed in the device respond appropriately to glucose within a time frame compatible with closed-loop insulin delivery, which requires a response time of  $<15$  min (34, 35); pharmacokinetic modeling of glucose homeostasis in man suggests that the lag time of the increase in insulin delivery by an artificial pancreas must be shorter than this to avoid the overexcursion of postprandial blood glucose (35). Indeed, the increase in insulin release *in vitro* in response to glucose from perfused macroencapsulated islets was delayed by only 7 min in comparison to nonencapsulated islets. Furthermore, glucose clearance during intravenous

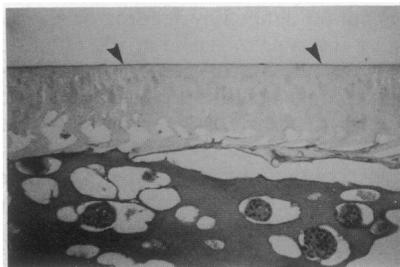


FIG. 9. Encapsulated canine islets retrieved from the peritoneal cavity of a diabetic rat 220 days after xenotransplantation. (Hematoxylin/eosin;  $\times 270$ .) Note the smooth skin on the external surface (arrowheads) versus the rough—i.e., the open trabecular or honeycombed surface—on the luminal side.

glucose tolerance testing was also not delayed. Since there is increasing evidence that the incidence and severity of the microvascular complications of diabetes are linked to the degree of metabolic control attained, the ability of the encapsulated islets to achieve rapid glucose-insulin kinetics may have important implications in assessing the potential role for this type of diffusion-based biohybrid artificial pancreas as therapy for human insulin-dependent diabetes.

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