Survival and Maturation of Microencapsulated Porcine Neonatal Pancreatic Cell Clusters Transplanted into Immunocompetent Diabetic Mice

Abdulkadir Omer, Valérie F. Duviyar-Kali, Nitin Trivedi, Karen Wilmot, Susan Bonner-Weir, and Gordon C. Weir

Differentiation and maturation of porcine neonatal pancreatic cell clusters (NPCCs) microencapsulated in barium alginate were assessed after transplantation into immunocompetent mice. Microencapsulated NPCCs were transplanted into the peritoneal cavity of streptozotocin-induced diabetic B6AF1 mice (n = 32). The microcapsules were removed at 2, 6, and 20 weeks and examined for cellular overgrowth, insulin content, and insulin secretory responses to glucose and glucose with theophylline. The differentiation, maturation, and proliferation of the β-cells in the NPCCs were assessed by immunohistochemistry. Blood glucose levels were normalized in 81% of the animals that received a transplant and remained normal until termination of the experiments at 20 weeks. Hyperglycemic blood glucose levels after transplantation of the capsules confirmed the function of the encapsulated NPCCs. Insulin content of the encapsulated NPCCs was increased 10-fold at 20 weeks after transplantation compared with pretransplantation levels. A 3.2-fold increase of the ratio of the β-cell area to the total cellular area was observed at 20 weeks, demonstrating the maturation of NPCCs into β-cells. In conclusion, NPCCs encapsulated with simple barium alginate can differentiate into β-cells and reverse high blood glucose levels in immunocompetent mice without immunosuppression for >20 weeks. *Diabetes* 52:69–75, 2003

As a result of recent progress (1), there is increased interest in islet transplantation as a potential therapy for type 1 diabetes. However, two major barriers must be overcome before islet transplantation can be provided for more patients: 1) the limited availability of human pancreatic tissue and 2) the need for permanent immunosuppression to prevent graft rejection and autoimmune (2). Xenogeneic islets from pigs and cows (3–5) have been considered as potential sources of islets for transplantation. Many factors favor the use of pigs: the similar structure of porcine and human insulin, the comparable glucose levels, and that both pigs and humans are omnivores. Islet cells can be isolated in large numbers from adult (6–9) or neonatal pigs (10,11). However, adult pig islets have proved to be difficult to isolate and tend to fare poorly in tissue culture, which has limited their use. Neonatal pancreatic cell clusters (NPCCs) contain a high proportion of islet precursor cells, can be maintained in culture, and differentiate into β-cells after transplantation (11). Naked (10,12) or microencapsulated NPCCs (13) have been shown to restore normoglycemia after transplantation into streptozotocin (STZ)-diabetic nude mice.

The concept of a bioartificial pancreas, consisting of islets enclosed within immunobarrier membranes, provides a potential way to overcome the need for immunosuppression. Our group recently developed a promising encapsulation method that uses highly purified alginate cross-linked with BaCl₂ without a separate permselective barrier, which protects islets against allorejection and autoimmunity (14). The aims of this study were to assess the protective capacity of simple barium-alginate capsules in a xenotransplantation model of NPCCs transplanted into STZ-induced diabetic immunocompetent mice and then to evaluate the growth, maturation, and function of these transplanted microencapsulated NPCCs.

RESEARCH DESIGN AND METHODS

Preparation of NPCCs. NPCCs were prepared using a modification (11) of a technique described by Korbett et al. (10). Male Yorkshire pigs (1–3 days old; Parsons Farm, Hadley, MA) were anesthetized with 0.15 ml of Telazol (tiletamine HCl; Fort Dodge Laboratories, Ft. Dodge, IA) and 0.30 ml of Xylaject (xylazine HCL; Phoenix Pharmaceutical St. Joseph, MO) given by intramuscular injection. After partial exsanguination, the pancreas was removed through a midline incision. Pancreata were then minced into 1- to 2-mm² pieces in a 50-ml plastic Falcon centrifuge tube containing 25 ml of M199 medium. After centrifugation for 1 min at 600 rpm, the supernatant was removed and 5 mg/ml collagenase (collagenase P; Roche, Indianapolis, IN) was added to the pellet. The pancreata were digested in a water bath at 37°C. After filtration, NPCCs were washed twice and were cultured in Ham's F10 media (Life Technologies, Grand Island, NY) supplemented with 10 mM l-glucose, 10 mM 3-isobutyl-1-methylxanthine (Sigma, St. Louis, MO), 2 mM l-glutamine (Life Technologies), 10 mM nicotinamide (Sigma), 25 mg/l CaCl₂.H₂O (Sigma), 0.5% BSA (RIA grade Fraction V, Sigma), and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, Life Technologies) in 150 x 50 mm bacteriological plates (Becton Dickinson, Franklin Lakes, NJ) at 37°C for 8 days. Media changes were performed on days 2, 4, 5, and 7.

Microencapsulation. Encapsulation was performed with a minor modification of a previously described technique (15), using a highly purified alginate containing 61% mannuronic acid and 39% guluronic acid (High M alginate 3.3%, the gift of John Holahan, Pharmacia, Peapack, NJ). At day 8 after preparation, NPCCs were washed with M199 medium and calcium-free Krebs Ringer buffer.
The number (given as islet equivalent [IE]) and the viability (determined with flow cytometry) of the NPCCs were determined after transplantation. The number of total capsules, and dithizone (dimethylthiocarbazone; Sigma) staining. Microencapsulated NPCCs were produced by crosslinking alginate molecules with a solution of CaCl₂ (20 mmol/l), which cross-links alginate molecules. Microencapsulated NPCCs were then cultured overnight in serum-free medium (Ultra-culture; BioWhittaker, Walkerville, MD) supplemented with penicillin-streptomycin and glutamine, as described above, at 37 °C in an atmosphere containing 5% CO₂. NPCCs from nine preparations were used for transplantsations.

Animals. Male B6AF1 mice (6–7 weeks old; Jackson Laboratories, Bar Harbor, ME) were used as recipients of nonencapsulated (controls) or microencapsulated NPCCs. One week before transplantation, animals were made diabetic by intraperitoneal injections (180 mg/kg body wt) of STZ (Sigma). Only animals with blood glucose levels >350 mg/dl on two consecutive measurements were used as recipients.

Transplantation of nonencapsulated NPCCs under the kidney capsule. Under methoxyflurane (Metofane; Shering-Plough Animal Health, Union, NJ) anesthesia, which was carried out in a 350-ml jar containing evaporated methoxyflurane (produced by gauze pads being soaked in the 3–5 ml of the liquid anesthetic in the jar), NPCCs were transplanted under the capsule of the left kidney by using PE-50 tubing (Becton Dickinson) connected to a 1-ml Hamilton syringe as previously described (11). Each mouse received 10,000 IE of NPCCs.

Transplantation of microencapsulated NPCCs. After washing with Ultra-culture medium, microcapsules were aliquotted and the volume of the capsules in each aliquot was determined in a 3-ml syringe (Becton Dickinson). Microencapsulated NPCCs were collected in a sterile transfer pipette and transplanted into the peritoneal cavity of Metofane-anesthetized mice (n = 32) through a small abdominal incision. Each mouse received 10,000 IE of NPCCs contained in 2.25 ± 0.06 ml of microcapsules.

Follow-up and intravenous glucose tolerance test. The body weight and the blood glucose levels of the mice were monitored twice weekly between 9:00 and 11:00 a.m. Glucose levels were measured with a portable glucometer (Precision QID; Medisense, Bedford, MA) on blood samples obtained from snipped tails. The mice were considered normoglycemic when blood glucose levels were <200 mg/dl. Twenty weeks after transplantation, an intravenous glucose tolerance test (IVGTT) (1.5 g/kg body wt) was performed in normoglycemic-transplanted mice and in body weight matched (having similar body weight but being at younger age than mice that received a transplant) and age-matched nondiabetic animals. Blood glucose samples were taken before and 2, 5, 10, 15, 30, 60, 90, 120, and 180 min after a glucose solution (30%) was injected through the orbital sinus of nonanesthetized mice. Blood samples for plasma C-peptide assessments were taken at the same time points except 2, 5, and 15 min after glucose injection.

Retrieval of capsules and overgrowth assessment. At 2, 6, and 20 weeks after transplantation, microcapsules were removed under Metofane anesthesia (Becton Dickinson) by using warmed Ringer HEPES buffer. The abdomen was sutured, and blood glucose levels were monitored during the next 2 weeks. The removed capsules were assessed for volume (in a 3-ml syringe), degree of fibrotic overgrowth (under a phase contrast microscope by systematic examination of 500 consecutive capsules for each mouse), structural integrity (by counting the number of the broken capsules as percentage of total capsules), and dithizone (dimethylthiocarbazone; Sigma) staining. Images were captured by a Magnafire digital camera (Optronics, Goleta, CA) connected to a microscope (CK2, Olympus, Tokyo, Japan) and are stored as.tif files.

Preparation of microencapsulated NPCCs for histology. Microencapsulated NPCCs were fixed in 10% buffered formalin (Fisher Scientific, Fairlawn, NJ) for 3 h and then stored in 0.1 mol/l Sorenson’s buffer. NPCCs were separated from alginate gel by incubation in 1 mol/l sodium citrate and finally in media containing 16.7 mmol/l glucose. NPCCs were washed three times with RPMI 1640 media without glucose (Life Technologies) supplemented with 10% neonatal calf serum for 3 h. Because of the anticipated low insulin output from NPCCs as a result of their immaturity in the pretransplantation period, microcapsules were incubated sequentially for 12 h as opposed to more conventional 1-h time periods. Microcapsules were first incubated in media containing 2.8 mmol/l glucose, then for 12 h in media containing 16.7 mmol/l glucose, and finally in media containing 16.7 mmol/l glucose + 10 mmol/l theophylline (Sigma). At the end of each incubation period, supernatant medium was collected and kept at −20 °C until measurement of insulin with a radioimmunoassay kit (Linco Research, St. Charles, MO). In addition, insulin content of microencapsulated NPCCs was measured for samples from two consecutive transplantsations. NPCCs were added to 0.5 ml of acid-ethanol solution, sonicated, and kept overnight at 4 °C. After centrifugation, supernatants were kept at −20 °C until measurement. Results are given as nanograms of insulin per IE.

Statistical analysis. Results are expressed as means ± SE. For the static incubations, results are the mean (nanograms of insulin per IE per 12 h) of triplicate samples measured for each animal at pretransplantation and 2, 6, and 20 weeks after transplantation. The insulin response to glucose and glucose plus theophylline was expressed as a stimulation index defined as the ratio of the insulin secretion at 16.7 mmol/l glucose and at 16.7 mmol/l glucose + 10 mmol/l theophylline over the insulin secretion at 2.8 mmol/l glucose. Student’s t tests for paired and unpaired data were used to compare data for IVGTT. Statistical analysis for histological insulin content and insulin response was performed with one-way ANOVA.

RESULTS

Encapsulation prolongs the survival of NPCCs for up to 20 weeks. Mice that received a transplant of 10,000 nonencapsulated (naked) NPCCs under the kidney cap-
weight: 20.7 ± 2.3 g before transplantation vs. 30.9 ± 2.9 g 20 weeks after transplantation (Fig. 1A). In the mice with normalized blood glucose levels (n = 26), the weight gain was 11 ± 1% at 2 weeks, 20 ± 1% at 6 weeks, and 51 ± 2% at 20 weeks compared with pretransplantation levels. Whereas 26 of 32 transplanted mice (81%) normalized blood glucose within 15 ± 12 days (range from 3 to 27 days; Fig. 1B), 6 mice from two preparations (of nine total preparations) did not reverse hyperglycemia, even at 20 weeks. It should be noted that none of the unsuccessful transplants with naked NPCCs used NPCCs from the apparently substandard preparations that never reversed hyperglycemia in the encapsulated group. Normoglycemia was maintained over the 20-week period in all other mice. After removal of capsules at 2, 6, and 20 weeks, animals (7, 7, and 12 mice, respectively) returned to hyperglycemic levels, showing the efficacy of the microencapsulated NPCCs.

**Mice that received a transplant of microencapsulated NPCCs have lower blood glucose levels during IVGTT.** An IVGTT was performed in all mice that received a transplant of microencapsulated NPCCs (20 weeks after transplantation) and body weight– and age-matched nondiabetic controls (Fig. 2). The fasting blood glucose level of mice that received a transplant of encapsulated NPCCs was 100 ± 21 mg/dl, which was similar to body weight–matched controls (102 ± 13 mg/dl) and lower than age-matched controls (138 ± 7 mg/dl). In all groups, blood glucose levels peaked at 2 min and started decreasing at 15 min. From this time point to the end of the IVGTT, significantly lower glucose levels were observed in the mice that received a transplant of microencapsulated NPCCs compared with the control groups (P < 0.05 at 15 min and P < 0.001 thereafter; Fig. 2).

**Microencapsulated NPCCs are functional and mainly free of overgrowth.** Capsules were removed 2, 6, and 20 weeks after transplantation and were incubated in the presence of different concentrations of glucose to assess the function of the removed capsules. Increased insulin secretory responses to 16.7 mmol/l glucose (0.09 ± 0.03, 0.26 ± 0.03, 0.32 ± 0.05, and 0.69 ± 0.1 ng ⋅ IE⁻¹ ⋅ 12 h⁻¹ at pretransplantation and 2, 6, and 20 weeks, respectively) and 16.7 mmol/l glucose plus theophylline (0.26 ± 0.05, 0.4 ± 0.04, 1.3 ± 0.3, and 2 ± 0.6 ng ⋅ IE⁻¹ ⋅ 12 h⁻¹ at the same time points) compared with insulin responses to 2.8 mmol/l glucose (0.06 ± 0.03, 0.15 ± 0.03, 0.18 ± 0.09, and 0.30 ± 0.04 ng ⋅ IE⁻¹ ⋅ 12 h⁻¹ at comparable time points) were observed. The stimulation index of encapsulated NPCCs in response to glucose alone increased compared with pretransplantation levels (1.1 ± 0.1 before transplantation; 2.3 ± 0.06 [P < 0.001], 2.9 ± 1.2 [P < 0.05], and 2.6 ± 0.2 [P < 0.001] at 2, 6, and 20 weeks, respectively; Fig. 3). A more pronounced increase in the stimulation index was observed with stimulation of glucose and theophylline (2.7 ± 0.5 before transplantation; 3.7 ± 1.1 at 2 weeks [P < 0.05], 10 ± 2.2 at 6 weeks [P < 0.001], and 9.4 ± 1.8 [P < 0.001] at 20 weeks).

The recovery percentage of capsules, corresponding to the ratio of the total capsular volume recovered over the volume before transplantation, was 91 ± 6, 85 ± 6, and 93 ± 2%, respectively at 2, 6, and 20 weeks, showing the stability of the Ba-alginate microcapsules. The majority of capsules were free of overgrowth at 2 and 20 weeks, with the percentage of clean capsules being 87 ± 11 and 80 ±
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5%, respectively. However, at 6 weeks, 43 ± 9% of the capsules were moderately to severely overgrown (Fig. 4).

DISCUSSION

Ilet microencapsulation, first described in 1980 (20), could represent a valuable therapeutic approach for the treatment of diabetes. This technique allows transplantation of islets enclosed in a capsule without immunosuppression, thus creating the potential to use xenogeneic islets. Previous studies have demonstrated protection of encapsulated pig islets from immune destruction and long-term maintenance of normoglycemia without immunosuppression. Lanza et al. (21) showed that transplantation of microencapsulated adult pig islets, enclosed in poly-L-lysine (PLL)-coated alginate capsules, could normalize the blood glucose levels of STZ-induced diabetic mice for 10 weeks. Furthermore, Murakami et al. (22) showed that encapsulation with agarose prolongs the survival of NPCCs for 6–8 weeks after transplantation in BALB/c mice. Sun et al. (4) reported long-term survival of microencapsulated adult porcine islets transplanted into STZ-diabetic Cynomolgus monkeys, although these results have yet to be reproduced.

In this study, we assessed the protective role of microcapsules made of highly purified high-M alginate cross-linked with BaCl2 without an additional permselective PLL coating. We cannot fully explain why these simple capsules work so well for islet xenografts. Previous studies have shown that barium cross-linkage provides stronger alginate gels than those found with calcium (23). In addition, the avoidance of PLL is probably helpful because the macrophage activation so often seen with PLL might alter the biocompatibility and stability (14). In addition, fabrication of microcapsules with highly purified and very low endotoxin-containing alginate probably contributed to the good results of our study. Finally, although not well documented, the mice used in these experiments may have a weaker reaction to xenografts than other species. Our results show that microencapsulated NPCCs sustained normoglycemia for >20 weeks in the majority of the mice.

FIG. 3. Microencapsulated NPCCs are functional as demonstrated by increased insulin secretory responses to glucose and theophylline during a 12-h static incubation. Triplicate determinations of secretion, each having 20 capsules, were performed for each of six mice at each time point. Starting from the second week, the insulin responses to glucose (open bars) and to glucose plus theophylline (black bars) were increased, reaching the highest point at 6 weeks. *P < 0.05 and **P < 0.001 versus pretransplantation secretion.

FIG. 4. Microencapsulated NPCCs are mainly free of overgrowth. Quantification of the overgrowth reaction on the surface of capsules was assessed on 500 microcapsules removed from each animal. The majority of capsules were free of overgrowth at 2 (n = 7) and 20 weeks (n = 9) with clean capsule ratios of 87 ± 11 and 80 ± 5%, respectively. However, at 6 weeks (n = 7), 43 ± 9% of the capsules were moderately to severely overgrown. The degree of overgrowth was scored on a semiquantitative basis from 0 to 3+: 0, clean capsules (no cells attached); 1+, ~100 cells were observed on the surface of the capsule; 2+, moderately overgrown capsules (less than half of the capsule was covered with cells); 3+, severely overgrown (more than half of the capsule covered with cells). The results were expressed as a percentage of total examined capsules.
that received a transplant. All of the mice that initially reversed hyperglycemia maintained normoglycemic limits until end of the experiments at 2, 6, and 20 weeks. Reversal of hyperglycemia after explantation of the capsules demonstrated the efficacy of encapsulated NPCCs. Six mice that did not become normoglycemic after transplantation all received NPCCs from the two preparations. These preparations of NPCCs had lower insulin content than the other preparations, which were successful. The factors responsible for batch-to-batch variation with NPCCs are unknown, but any number of the multiple variables could be involved.

The period of normalization of the glycemia after transplantation (15 ± 12 days; range 3–27 days) was shorter than has been observed in different studies in which nonencapsulated (10,11,19) or encapsulated (13) NPCCs were transplanted into diabetic nude mice. However, our results are consistent with the study of Murakami et al. (22), in which glucose levels were normalized 3–4 weeks after transplantation of 8,000 encapsulated NPCCs in the peritoneal cavity of diabetic mice. It is noteworthy that the study of Rayat et al. (13) found that only 2,000 encapsulated NPCCs could reverse diabetes in nude mice, with this result being better than that obtained with similar numbers of unencapsulated NPCCs. However, in other studies, the encapsulation approach seems to require larger numbers of either islets or NPCCs to normalize glucose levels (11,14,19,22). The reasons that so many NPCCs are required are that the peritoneal cavity is not a particularly efficient site for transplantation and NPCCs contain precursor cells and immature islets, which limit insulin production. In our preliminary studies, 5,000 IE of encapsulated NPCCs were unable to produce normoglycemia (A.O., unpublished data). The capsules used in this study were relatively large at 0.8–1.0 mm in diameter, but this diffusion distance did not seem to prevent adequate provision of nutrients, glucose, and oxygen. It is noteworthy that glucose clearance after intravenous glucose was much faster than in control mice. This may be because porcine islets have a lower set point for glucose-induced insulin secretion than mouse islets, which would make the mice relatively hyperinsulinized. We were surprised, however, by the slow rate of clearance in the control mice compared with experiments in which younger mice of a different strain were used (14).

In assessing the immune reaction on the microcapsules after removal, it was found that at 2 and 20 weeks after transplantation, the majority of the microcapsules (87–90%, respectively) were free of any fibrotic or cellular reaction, indicating the biocompatibility of the uncoated capsules. Unexpectedly, 6 weeks after transplantation, IgG was found to accumulate on the alginate capsules (Fig. 6). Immunostaining was performed without primary antibody, confirming that secondary antibody (FITC-conjugated anti-mouse antibody) binds to IgG that accumulated in vivo on the alginate and on the NPCCs (arrow indicates the thick layer of the IgG on the NPCC, and red staining represents insulin). Bar = 100 μm. Therefore, polyclonal antibodies were used for CK and Ki-67 immunostaining.
tation in cured animals, the majority of the capsules had a mild to moderate reaction on their surface. This result raises interesting questions about the amount of shed antigens and the time of shedding required to develop an immune reaction on the microcapsules, as well as the possibility of a later accommodation of the immune system to the presence of the encapsulated xenogeneic islets. It has been shown that a strong immune reaction to the nonencapsulated fetal porcine islet cell clusters occurs during the first week of transplantation (24). In addition to the cellular infiltrate, we observed IgG deposition on the NPCCs contained in the microcapsules at 6 and 20 weeks after transplantation (Fig. 6, 6-week example not shown). These results support the idea that IgG can diffuse through the capsule and yet not exert any adverse effect on the encapsulated NPCCs. Similar observations have been reported by Friedman et al. (25) who found that rejection of fetal islet cell clusters seemed to be mediated only by CD4+ cells without a contribution from IgG.

The second aim of this study was to assess the proliferation and differentiation of encapsulated NPCCs after transplantation. NPCCs contain more mature islet cells than fetal porcine islet clusters, yet they still possess considerable capacity for further differentiation, thus providing a potentially valuable source of β-cells for transplantation (10). The present results demonstrate maturation of NPCCs contained in the capsules as evidenced by graft insulin content and immunostaining results. We observed a 12-fold increase in the graft insulin content at 6 weeks, with a similar increase at 20 weeks compared with pretransplantation insulin content. These results are similar to those observed with nonencapsulated NPCCs transplanted in the subrenal space of nude mice (10,11,19), in which it seems that the increase in β-cell mass after transplantation was due to both proliferation of β-cells and differentiation of duct cells into β-cells. Although we were not able to quantify duct cells with specific staining for CK7 at 6 and 20 weeks because of the interference from IgG deposition, we showed a significant increase in the β-cell area, which we assume results from a developmental pattern similar to that which occurs with nonencapsulated NPCCs.

To our knowledge, this is the first study to show differentiation of encapsulated duct cells and expansion of β-cells in the NPCCs after transplantation into immunocompetent mice. As was the case with nonencapsulated NPCCs (19), we found a high β-cell proliferation index before transplantation and 6 weeks afterward, with a decline at 20 weeks. However, those results are not in agreement with another study that showed increased proliferation index and apoptosis of encapsulated rat islets followed by a fall in β-cell mass (26). Discrepancies in results may be due to different encapsulation models (PLL versus non-PLL), differences in the maturity of islets, and species differences. We do not know how long encapsulated NPCCs can continue to function, but at 20 weeks after transplantation, they seem to be stable and healthy.

In conclusion, these results indicate that an alginate barrier, without a traditional permeselective membrane, can provide protection against xenorejection in mice. Furthermore, the encapsulated NPCCs can differentiate into β-cells. These results provide support for the idea that neonatal porcine pancreas could provide large numbers of insulin-producing cells for transplantation (2).

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