

# Why Do Microencapsulated Islet Grafts Fail in the Absence of Fibrotic Overgrowth?

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The survival of microencapsulated islet grafts is limited, even if capsular overgrowth is restricted to a small percentage of the capsules. In search of processes other than overgrowth contributing to graft failure, we have studied the islets in non-overgrown capsules at several time points after allotransplantation in the rat. All recipients of islet allografts became normoglycemic. Grafts were retrieved at 4 and 8 weeks after implantation and at  $15.3 \pm 2.3$  weeks postimplant, 2 weeks after the mean time period at which graft failure occurred. Overgrowth of capsules was complete within 4 weeks postimplant, and it was usually restricted to <10% of the capsules. During the first 4 weeks of implantation, 40% of the initial number of islets was lost. Thereafter, we observed a decrease in function rather than in numbers of islets, as illustrated by a decline in the ex vivo glucose-induced insulin response. At 4 and 8 weeks postimplant,  $\beta$ -cell replication was 10-fold higher in encapsulated islets than in islets in the normal pancreas, but these high replication rates were insufficient to prevent a progressive increase in the percentage of nonviable tissue in the islets. Necrosis and not apoptosis proved to be the major cause of cell death in the islets. The necrosis mainly occurred in the center of the islets, which indicates insufficient nutrition as a major causative factor. Our study demonstrates that not only capsular overgrowth but also an imbalance between  $\beta$ -cell birth and  $\beta$ -cell death contributes to the failure of encapsulated islet grafts. Our observations indicate that we should focus on finding or creating a transplantation site that, more than the unmodified peritoneal cavity, permits for close contact between the blood and the encapsulated islet tissue. *Diabetes* 48:1381-1388, 1999

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Received for publication 23 March 1998 and accepted in revised form 22 March 1999.

BrdU, bromo-2-deoxyuridine; BSA, bovine serum albumin; FCS, fetal calf serum; GMA, glycol methacrylate; KRBB, Krebs-Ringer bicarbonate buffer; KRH, Krebs-Ringer supplemented with 25 mmol/l HEPES buffer; PLL, poly-L-lysine; TDT, terminal deoxynucleotidyl transferase.

The introduction of biocompatible materials has brought important advances in the immunoisolation technology during recent years. The first allotransplantations in humans with alginate-based microcapsules containing parathyroid cells have been successfully performed in the absence of immunosuppression (1). Also, a first allotransplantation of an islet graft in capsules has shown some degree of success in an immunosuppressed type 1 diabetic patient (2). In the absence of immunosuppression, normoglycemia has been reported after transplantation of microencapsulated allo- and xenogeneic islets in both chemically induced and autoimmune diabetic rodents (3,4), dogs (5), and monkeys (6). Although this illustrates the principal applicability of encapsulated islet transplantation, a major and probably fundamental barrier has to be overcome for clinical application on a larger scale, since graft survival was always limited to several months and never indefinite.

Graft failure of encapsulated islets cannot be explained by rejection, since the capsules have been shown to adequately protect the tissue for effector molecules during immune attack (7-9). Another observation excluding rejection as a causative factor is the similar survival rates of islet isografts and islet allografts (7). Usually, the limited graft survival is interpreted as the consequence of a nonspecific foreign body reaction against the microcapsules resulting in progressive fibrotic overgrowth of the capsules (10-14) and necrosis of the islets. However, when islets in biocompatible capsules are retrieved after graft failure, not more than 10% of the capsules shows overgrowth by fibroblasts and macrophages (10, 15-17). Thus, encapsulated islets cease to function, in spite of the fact that the major part of the capsules is freely floating without fibrotic overgrowth. Obviously, processes other than overgrowth with subsequent necrosis contribute to cessation of graft function.

In search of these processes, we have studied the islets contained by these non-overgrown capsules at several time points after intraperitoneal allotransplantation in the rat. The number and volume of islets, their ex vivo response to glucose, the rate of islet cell proliferation, and the percentage of nonviable (necrotic or apoptotic) tissue in the islets were determined after peritoneal lavage at 4 or 8 weeks after transplantation into both normoglycemic and hyperglycemic recipients or at 2 weeks after the recurrence of hyperglycemia. At each time point, the recovery rates of the capsules implanted and the percentage of recovered capsules showing fibrotic overgrowth were systemically determined to assess

the contribution of fibrotic overgrowth and subsequent islet cell necrosis or apoptosis to the limited duration of encapsulated islet graft function. This approach enabled us to evaluate the rate of decline in function and viability of the islets in non-overgrown capsules and the role of an unfavorable balance between proliferation and cell death. Thereby, our study provides insight to the causative factors responsible for the limited survival of an encapsulated islet graft. The results will serve to design strategies to prolong the survival of encapsulated islet grafts.

## RESEARCH DESIGN AND METHODS

**Animals and induction of diabetes.** Male inbred Lewis rats (Harlan CPB, Zeist, the Netherlands) weighing 300–350 g served as donors. Male inbred Albino Oxford rats (AO/G; the Central Animal Laboratory of Groningen, the Netherlands) weighing 290 to 320 g were used as recipients of encapsulated islet grafts. Diabetes was induced by injection of 75–90 mg/kg of streptozotocin (Zanosar, Upjohn, Kalamazoo, MI) via the tail vein. Glucose concentration in blood was determined with glucose test tapes (Reflux; Boehringer Mannheim, Mannheim, Germany). A second injection of 70–90 mg/kg of streptozotocin was administered if the blood glucose level was lower than 20 mmol/l at 10 to 14 days after the first injection. Only animals with severe weight loss, polyuria, polydipsia, and blood glucose levels >20 mmol/l over a period of at least 4 weeks were used as recipients. To exclude  $\beta$ -cell regeneration, we always took a biopsy from the pancreas at the time of peritoneal lavage. The absence of  $\beta$ -cells in the native pancreas, defined as <5% of normal controls, was always histologically confirmed at the time of death.

**Islet isolation and culture.** Islets were isolated as previously described (18). Briefly, under ether anesthesia, the abdomen was opened and the common bile duct was cannulated under nonsterile conditions. The donor pancreas was distended with 10 ml sterile Krebs-Ringer-HEPES supplemented with 25 mmol/l HEPES buffer (KRH) and containing 10% bovine serum albumin (BSA). Subsequently, the pancreas was excised and brought into a laminar flow cabinet. All further procedures were performed under sterile conditions.

The pancreas was chopped, digested using a 20-min, two-stage successive incubation at 37°C with 1.0 and 0.7 mg/ml collagenase (type XI; Sigma, St. Louis, MO). Islets were separated from exocrine tissue by centrifugation over a discontinuous Dextran gradient (19,20) and further purified by handpicking.

The total islet volume was determined by measuring the diameters of islets in a 4% aliquot of the islet suspension (21). The diameters were measured with a dissection microscope with a fluorescent illuminator (Bausch and Lomb BVB-125 and 31-33-66) equipped with an ocular micrometer with an accuracy of 25  $\mu$ m. The total volume was calculated, assuming the islets to be perfect spheres. In each experiment, we used four to five donors from which we isolated 3,500–4,200 islets, which corresponds to 10–15  $\mu$ l of islet tissue.

Islets were cultured before encapsulation to reduce contamination of the grafts with exocrine tissue, ducts, or damaged cells. They were cultured in non-treated petri dishes (Greiner, Alphen a/d Rijn, the Netherlands) in portions of 100 islets per 25 cm<sup>2</sup> for 19 to 44 h in CMRL1066 containing 10% fetal calf serum (FCS) (Gibco), 8.3 mmol/l glucose, 10 mmol/l HEPES, and 1% penicillin/streptomycin) at 37°C in humidified air containing 5% CO<sub>2</sub>. After this culture period, the total islet volume was assessed again in a 5% aliquot of the islet suspension. Graft endocrine volume was at least 10  $\mu$ l, which is approximately equal to the endocrine volume of the normal rat pancreas (21).

**Islet encapsulation.** Purification of alginate (Keltone LV; Kelco International, London, U.K.) was performed as described in detail by our group elsewhere (7). Briefly, alginate was dissolved in a 1 mmol/l EGTA solution to a 1% solution and successively filtered over 5.0-, 1.2-, 0.8-, and 0.45- $\mu$ m filters (Schleicher and Schüll, 's-Hertogenbosch, The Netherlands). During this step, all visible aggregates were removed. Next, the pH of the solution was gradually lowered to 2.0, which induces precipitation of alginate as alginate acid. To remove nonprecipitated contaminants, the precipitate was brought into a Buchner funnel and vigorously washed with 0.01 N HCl plus 20 mmol/l NaCl. Then proteins were removed by extraction with a chloroform/butanol solution (16% chloroform and 4% butanol in 0.01 N HCl plus 20 mmol/l NaCl). This extraction was repeated after dissolving the alginate acid in water by gradually increasing the pH to 7.0. Finally, the alginate was precipitated by adding 200 ml absolute ethanol to each 100 ml alginate solution and freeze dried. The alginate was dissolved in 220 mosm Ca<sup>2+</sup>-free Krebs-Ringer-HEPES solution to a 3–3.3% alginate solution to obtain a solution with a viscosity of 4.3 cps. This viscosity is necessary for the production of perfect spherical droplets without any tails or other imperfections associated with graft failure. Before use, the alginate solution was sterilized by 0.2  $\mu$ m filtration. As previously reported (7), after purification the alginate was composed of 40% gluturonic and 60% manuronic acid.

The alginate was mixed with cultured islets to a concentration of 2,000 islets/ml. Higher numbers of islets per milliliter is associated with an increase of the number of islets protruding from the capsules (22,23). The islet-alginate mixture was converted into droplets using an air-driven droplet generator as previously described (24). Polylysine-alginate encapsulation was performed as described elsewhere (25). Briefly, the alginate droplets were transformed to alginate beads by gelling in a 100 mmol/l CaCl<sub>2</sub> (10 mmol/l HEPES, 2 mmol/l KCl) solution for at least 5 min. After gelling, the beads had a diameter of 450–550  $\mu$ m. Subsequently, the Ca-alginate beads were suspended for 1 min in KRH containing 2.5 mmol/l CaCl<sub>2</sub>. A poly-L-lysine (PLL) membrane was formed by suspending the alginate beads in 0.1% PLL solution for 10 min (poly-L-lysine-HCl, molecular weight 22,000; Sigma). Nonbound PLL was removed by three successive washings during 3 min with Ca<sup>2+</sup>-free KRH containing 135 mmol/l NaCl. The outer alginate layer was subsequently applied by 5-min incubation in 10-times diluted alginate solution. Usually, within one graft the variation in the diameter of the capsules was <2% and between 700 and 800  $\mu$ m. As a mean, the grafts had a diameter of 739  $\pm$  16  $\mu$ m. A mean of 53  $\pm$  4.1% of the capsules contained one or two islets. No attempts were made to remove the empty capsules, since we found that procedures to remove empty capsules such as handpicking or density-gradients were associated with more fibrotic overgrowth and, thus, a decrease in graft biocompatibility.

The total capsule graft volume to be implanted varied between 2.0 and 2.5 ml per islet graft, determined with a 5 ml measure cylinder, with an accuracy of 0.05 ml (7,10,16).

**Implantation and retrieval of the grafts.** The microencapsulated islets or empty capsules were injected into the peritoneal cavity with a 16-G cannula via a small incision (3 mm) in the linea alba. The abdomen was closed with a two-layer suture.

Peritoneal lavage was performed as previously described (7,10,16). Briefly, 20 ml prewarmed (37°C) RPMI was infused through a 3-cm midline incision into the peritoneal cavity and the abdomen was flushed above a 50 ml centrifuge tube. The abdominal organs were flushed two to three times with RPMI. The final volume of the retrieved capsules was assessed with the same technique as mentioned for assessment of the settled capsules graft volume (see above) and expressed as percentage of the initial volume of the implanted capsules. Subsequently, in a 25–50% aliquot of the retrieved capsules, we determined the number of retrieved islets and the diameters of those islets to calculate the volume of the islet graft (21). Since a change in diameter of the capsules may interfere with adequate measurement of the retrieved graft volume, we also measured the diameters of the capsules in a 25–50% aliquot of the retrieved graft and confirmed that the capsule diameter was identical before and after transplantation. The retrieved islet numbers and the volume of the islet graft were expressed as the percentage of the initial implanted number of islets and the initial implanted volume of the islet graft.

During each surgical procedure, the rats were anesthetized with halothane.

**Labeling of islets.** For bromo-2-deoxyuridine (BrdU) labeling of nonencapsulated islet cells in vitro, islets were cultured for 3 days and subsequently incubated for 24 h in culture medium containing 10  $\mu$ mol/l BrdU. For BrdU labeling of encapsulated islets in vitro, islets were cultured for 2 days, encapsulated, cultured for another day, and then incubated for 24 h in culture medium containing 10  $\mu$ mol/l BrdU. BrdU labeling was always performed in culture medium containing 15 mmol/l glucose, a concentration that stimulates islet cell replication (26).

To label replicating cells in vivo, a BrdU tablet (Boehringer Mannheim) was implanted subcutaneously via a small incision in the skin of the neck at exactly 24 h before peritoneal lavage.

**Ex vivo insulin secretion during glucose challenge.** Directly after peritoneal lavage, microencapsulated islets were tested in four separate samples of 25 islets for their glucose-induced insulin response. To minimize the variability of the mean insulin responses, we meticulously selected islets with diameters between 150 and 200  $\mu$ m. The encapsulated islets were preincubated for 45 min in 2 ml Krebs-Ringer bicarbonate buffer (KRBB), gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, containing 0.25% BSA and 2.75 mmol/l glucose. The ex vivo insulin secretion was then assessed by three consecutive incubations of 1) 45 min in 2.75 mmol/l glucose in KRBB, 2) 60 min in 16.5 mmol/l glucose in KRBB, and 3) 45 min in 2.75 mmol/l glucose in KRBB. We allowed a somewhat extended incubation time in high glucose because transplanted encapsulated islets (as opposed to fresh) (25) showed a somewhat delayed start of the insulin secretion with not more than a modest response at 15 min and an outright response during the subsequent 45 min. This was determined by taking samples every 15 min in pilot experiments (data not shown). We have used the 60 min data in high glucose to calculate the production of insulin per 45 min. The insulin secretory responses were expressed as nanograms of insulin per milliliter per 25 islets per 45 min.

At the end of each incubation, the incubation media were completely removed and frozen for insulin determination by a radioimmunoassay for rat insulin.

**Histologic procedures.** The various tissue samples—encapsulated tissue from in vitro studies and encapsulated tissue from in vivo studies—required a different type of histotechnical processing for optimal histo- and immunocytochemistry (27,28), as specified below.

**Assessment of overgrowth.** Nonadherent capsules recovered by peritoneal lavage were always divided in two portions. One portion was used for quantification of apoptosis (see below). The other portion was fixed in precooled 2% paraformaldehyde, buffered with 0.05 mol/l phosphate, and processed for glycol methacrylate (GMA) embedding (27,28). Sections were prepared at 2  $\mu$ m. Three to six of these sections were stained with Romanovsky-Giemsa and applied for determining the number of capsules with and without overgrowth and the percentage of necrotic tissue in the islets. The number of recovered capsules with fibrotic overgrowth was assessed by microscopy. The fraction of overgrown capsules was expressed as the percentage of the total number of recovered capsules.

Processing for GMA embedding and Romanovsky-Giemsa staining was also applied to samples of adherent capsules recovered by excision.

**Quantification of replication.** BrdU-labeled islets obtained from in vitro experiments were imbedded in tissue-teck and frozen in precooled 2-methylbutane. Sections were prepared at 5  $\mu$ m and processed for immunocytochemical staining of BrdU-positive islet-cells without staining for non- $\beta$ -cell hormones.

The approach of using frozen sections was abandoned for the subsequent larger set of in vivo experiments since we found that this method was associated with loss of islets during histochemical processing and with inadequate morphology for simultaneously counting BrdU-positive cells and confirming absence of overgrowth (that is, the capsules in which the number of BrdU-positive islet cells was assessed). Therefore, we applied GMA sections of the nonadherent capsules instead of frozen sections for identification of BrdU-positive cells in the  $\beta$ -cell and non- $\beta$ -cell fractions of the islets. To this end, we applied a double-staining protocol for the non- $\beta$ -cell hormones and BrdU as previously described (29,30). We stained the non- $\beta$ -cells instead of the  $\beta$ -cells since  $\beta$ -cells were expected to be degraded after graft failure. To validate this method, additional sections of encapsulated islets retrieved from four animals (at 4 and 8 weeks postimplant) were double-stained for BrdU and for the  $\beta$ -cell hormone insulin instead of for the non- $\beta$ -cell hormones (Fig. 1). The results of the two methods were compared and found to be similar. This confirms the results of Nacher et al. (31), who also found similar results with the two methods.

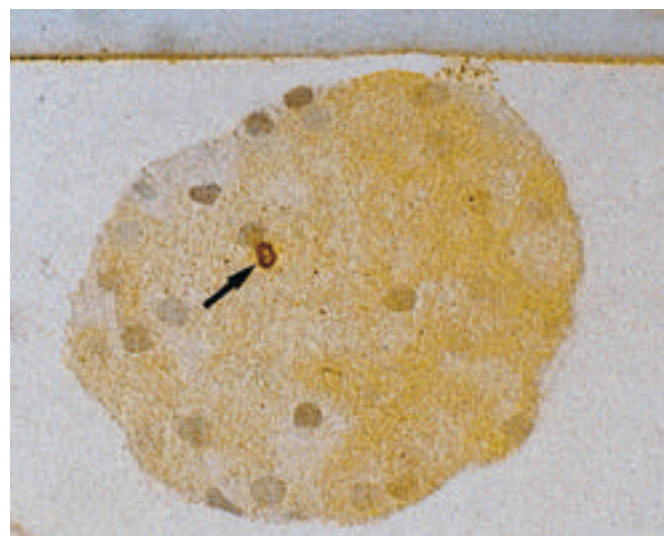
The sections were incubated with a cocktail of the following antibodies: guinea pig anti-swine insulin antibodies, rabbit anti-glucagon, rabbit anti-human somatostatin, and rabbit anti-human pancreatic polypeptide (all from DAKO, Santa Barbara, CA). After several washings, sections were incubated with a goat anti-rabbit, alkaline phosphatase-conjugated second-step reagent. Phosphatase activity was demonstrated by applying Naphthol As-MX phosphate disodium salt and Fast Blue BB salt. Next, the sections were pretreated with HCl for DNA denaturation and incubated with a mouse monoclonal antibody against BrdU. After several washing steps, the sections were incubated with rabbit anti-mouse, peroxidase-conjugated second-step reagent. To enhance the signal of the BrdU antibody, the sections were subsequently incubated with a goat anti-rabbit, peroxidase-conjugated third-step reagent. Peroxidase activity was demonstrated by applying 3-amino-9-ethylcarbazole (AEC) containing hydrogen peroxide. Finally, the sections were incubated, in sequence, in celestin blue and hematoxylin for staining the cell nuclei.

Per graft, we counted at least 900 cells, and per islet culture, we counted at least 500 cells. In some instances, we found cells in the necrotic area that were weakly stained for BrdU. These cells were excluded from counting since it cannot be excluded that the BrdU is incorporated during a process of repair and therefore indicates cell repair rather than replication. The number of BrdU-positive  $\beta$ -cells was expressed as the percentage of the total number of counted  $\beta$ -cells.

Unfortunately, it was not possible to quantify the percentage of BrdU-positive non- $\beta$ -cells in the encapsulated islets, since non- $\beta$ -cells were present only in low and insufficient numbers for reliable quantification. Another reason prohibiting the reliable quantification of the number of non- $\beta$ -cells was that about 0–5% of the cells had a very weak cytosolic staining. These cells were excluded from counting. Exclusion of this small portion of 0–5% cells has negligible consequences for the percentage of replicating  $\beta$ -cells.

Pancreas biopsies for determination of the number of BrdU-positive  $\beta$ -cells were fixed in 2% paraformaldehyde for GMA processing and processed for immunocytochemistry as described above.

**Assessment of necrosis.** Necrosis in the retrieved islets was assessed by identifying cells or groups of cells in the islets with the morphologic characteristics of necrosis. To this end, 40–100 recovered islets were microscopically examined to identify cells with a swollen appearance, cells with a loss of plasma integrity, and zones in the islets composed of cell-debris. The surface area was determined by measuring the diameters of the islets and their necrotic zone (21). The diameters were measured with a microscope (Leitz Orthoplan) with inverted phase contrast equipped with an ocular micrometer with an accuracy of 1.25  $\mu$ m. The surface area was calculated, assuming the islets and the necrotic zones to be perfect spheres (21). Subsequently, the necrosis in the islets was quantified by expressing the mean surface area of the islets composed of necrotic tissue as the percentage of the total surface area in each individual islet.



**FIG. 1. Replicating  $\beta$ -cells in a microencapsulated islet, 4 weeks after successful allotransplantation in a diabetic rat. At the time of peritoneal lavage, the animal was still normoglycemic. The arrow indicates a BrdU-labeled  $\beta$ -cell (GMA-embedded section, double-stained for the  $\beta$ -cell hormone insulin and BrdU, original magnification  $\times 200$ ).**

**Quantification of apoptosis.** Nonadherent capsules (derived from one or both portions as mentioned under assessment of overgrowth) were fixed in Bouin's solution for paraffin processing, sectioned at 5  $\mu$ m, and stained for non- $\beta$ -cell hormones as described above. Subsequently, apoptotic cells were immunostained by applying a modified terminal digoxinucleotidyl transferase (TDT) method (Apoptac; Oncor, Gaithersburg, MD). The apoptotic cells were identified by microscopy. A cell was classified as apoptotic when the nucleus was immunostained and had the characteristic morphology of an apoptotic cell, that is, a  $\beta$ -cell containing a condensed or fragmented nucleus. Islets in serial slices treated with DNase I (5 mg/ml, 20 s, 37°C) and lymph nodes in the pancreas served as positive controls. In addition, we quantified apoptosis by assessing the number of apoptotic bodies in GMA-embedded, Romanovsky-Giemsa-stained slices of encapsulated islets.

Pancreas biopsies for determination of apoptotic  $\beta$ -cells were fixed in Bouin's solution for paraffin processing, sectioned at 5  $\mu$ m, and processed for immunocytochemistry as described above. Other sections were stained with aldehyde fuchsin to determine the presence or absence of viable islet cells.

**Statistical analysis.** Statistical evaluation was performed using the Mann Whitney *U* test. Values are expressed as mean  $\pm$  SE. Only  $P < 0.05$  was considered to indicate a statistically significant difference.

## RESULTS

**Graft function and overgrowth of capsules.** All streptozotocin-induced diabetic AO recipients of encapsulated allogeneic islets became normoglycemic within 5 days after implantation. Grafts were retrieved by peritoneal lavage at 4 and 8 weeks after implantation and at  $15.3 \pm 2.3$  weeks postimplant (2 weeks after the mean time period at which graft failure occurred). Encapsulated allogeneic islets were implanted not only in diabetic but also in normoglycemic AO-recipients and retrieved by peritoneal lavage at 8 weeks postimplant.

At the time of peritoneal lavage, we occasionally found a portion of the capsules to be adherent to the omentum and the liver capsule. Most of the capsules, however, were freely floating in the abdominal cavity without adhesion to the abdominal wall or organs, as quantified by a mean retrieval rate of  $>85\%$  of the capsules.

Virtually all capsules adherent to the abdominal wall or organs were found to be overgrown by fibrotic tissue. However, of the  $>85\%$  of capsules retrieved by peritoneal lavage, the mean percentage of capsules with overgrowth was usually well below 10%. This percentage was not influenced by

TABLE 1

Recovery rates and percentage of capsules with overgrowth at varying time intervals after implantation of microencapsulated rat allografts in streptozotocin-induced diabetic AO rats

Time period between implantation and peritoneal lavage (weeks)	<i>n</i>	State immediately preceding implantation	State immediately preceding peritoneal lavage	Percentage of retrieval	Percentage of capsules with overgrowth
4	4	Hyperglycemia	Normoglycemia	91 ± 2.7	7.3 ± 2.5
8	5	Hyperglycemia	Normoglycemia	93 ± 3.7	9.0 ± 1.3
8	5	Normoglycemia	Normoglycemia	88 ± 5.8	15.4 ± 6.5
15.3	6	Hyperglycemia	Hyperglycemia	85 ± 11.9	8.3 ± 5.0

Data are means ± SE. Peritoneal lavage was performed immediately after death in the animals that were normoglycemic preceding peritoneal lavage. In the other recipients, it was performed 2 weeks after recurrence of hyperglycemia.

the time that the graft remained in the peritoneal cavity, since the percentages of overgrown capsules were similar at 4, 8, and 15.3 weeks after implantation (Table 1).

**Retrieval of islet-containing microcapsules.** At 4 weeks after implantation in diabetic recipients, 91 ± 2.7% of the capsules were retrieved (Table 1), in which we found not more than 61 ± 7.1% of the initially implanted number of islets. This percentage of explanted islets was somewhat lower at 8 weeks after transplantation, but the difference was not statistically significant (Fig. 2). The concomitant decrease of the calculated volume of the islet graft was even somewhat higher. At 4 weeks after transplantation, only 39 ± 4.8% of the volume had remained, and at the time of graft failure it had decreased to 22 ± 7.4% ( $P < 0.05$ ) of the initially transplanted islet graft volume (Fig. 2). This involution of the graft cannot be explained by exhaustion of the encapsulated islets in the diabetic recipients, since it was also observed in normoglycemic recipients in which, at 8 weeks after implantation, 88% of the capsules were retrieved containing 52 ± 5.3% of the initial islet number and 25 ± 3.6% of the initial islet volume.

The more pronounced decrease in volume than in numbers of islets (Fig. 2) is the consequence of a preferential disappearance of the larger islets in the graft (Fig. 3). Notably, these larger islets do not dissolve but rather experience a decrease in diameter, as illustrated by the concurrent observation of an increase in number of islets with

small diameters in the graft (Fig. 3) and an approximately equal number of retrieved islets at 4, 8, and 15.3 weeks after transplantation (Fig. 2).

**Insulin secretion by explanted islets.** As shown in Fig. 4, the explanted microencapsulated islets always responded to glucose stimulation, even after the occurrence of graft failure. However, the magnitude of the response gradually decreased with longer periods of time in the peritoneal cavity (Fig. 3). This gradual decrease in responsiveness was accompanied by an incomplete return to basal insulin secretion during the second incubation in low glucose. The difference between stimulated insulin levels and poststimulated levels was statistically significant at 4 weeks postimplant ( $P < 0.02$ ), but not more than a trend at 8 weeks postimplant and after graft failure (both  $P < 0.1$ ). In normoglycemic recipients, the responses were even somewhat lower than in the diabetic recipients.

**Replication, necrosis, and apoptosis in encapsulated islets.** Since the presence of a capsule around cells reportedly inhibits replication (32–34), we first determined whether our capsule has such an effect on islet cells. To this end, we compared the percentage of replicating islet cells in free and encapsulated islets, both derived from one islet isolation procedure in frozen sections. This experiment was repeated eight times. The mean percentage of 6.1 ± 0.8% in free islets was similar to the 6.5 ± 0.9% found in encapsulated islets.

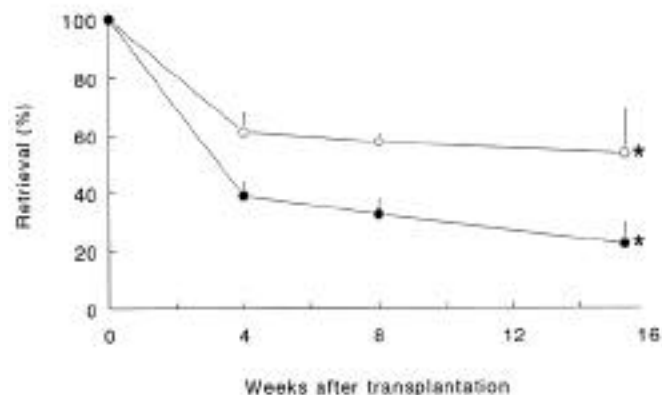


FIG. 2. Percentage of explanted islets (○) and the calculated volume of the explanted islet graft (●) at varying time intervals after transplantation. Asterisk indicates the mean time point of peritoneal lavage, which was performed 2 weeks after recurrence of hyperglycemia. The *n* values are similar to those given in Table 1. Values represent means ± SE.

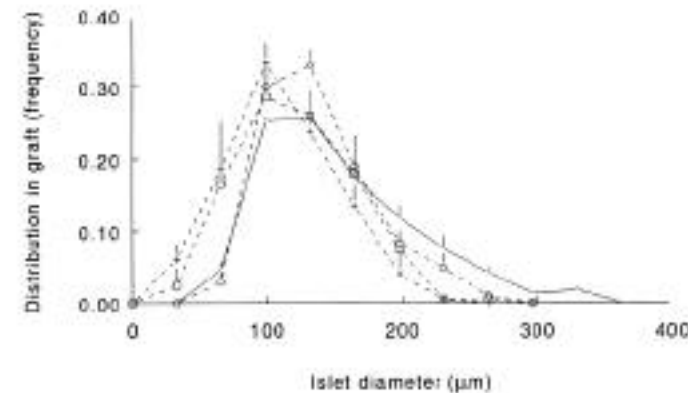


FIG. 3. Distribution of the islet diameters in the grafts before transplantation (—), at 4 (○) and 8 (□) weeks after transplantation, and at 2 weeks after occurrence of graft failure (+). After transplantation, the mean islet diameter was always significantly smaller than the mean islet diameter before transplantation ( $P < 0.02$ ). The *n* values are similar to those given in Table 1. Values represent means ± SE.

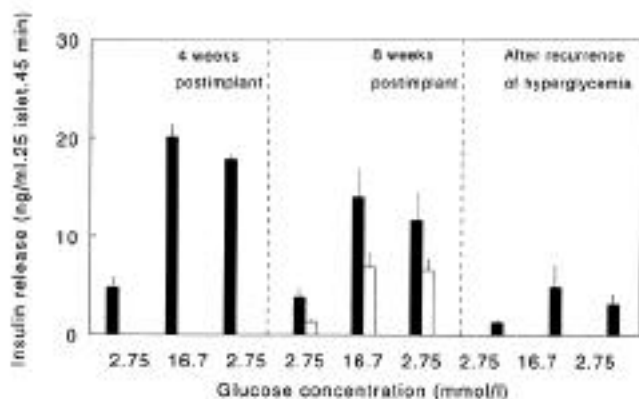


FIG. 4. Glucose-induced insulin response (ex vivo) of rat islets at 4 and 8 weeks after transplantation and at 2 weeks after the recurrence of hyperglycemia. Recipients were diabetic (■) or normoglycemic (□) preceding transplantation. The *n* values are similar to those given in Table 1. Values represent means  $\pm$  SE.

In islets obtained from the in vivo experiments, we specifically determined the percentage of replicating  $\beta$ -cells in GMA sections. The normal percentage of  $\beta$ -cell replication was determined in the pancreas of the nondiabetic recipients killed at 8 weeks after implantation of the encapsulated islets. As shown in Fig. 5,  $\beta$ -cell replication was higher in encapsulated islets at 4 ( $P < 0.02$ ) and 8 weeks ( $P < 0.02$ ) after implantation than in islets in the normal pancreas. After graft failure, however, the percentage of replicating  $\beta$ -cells was lower than in the functioning grafts, and it was not significantly higher than the percentage of replicating  $\beta$ -cells in the normal pancreas. The elevated  $\beta$ -cell replication in encapsulated islets at 4 and 8 weeks postimplant was not induced by metabolic demand (29,30), since elevated  $\beta$ -cell replication was also found in the nondiabetic recipients.

Surprisingly, we observed not only high numbers of replicating  $\beta$ -cells but also high numbers of replicating non- $\beta$ -cells in the encapsulated islets (Fig. 6). Replicating non- $\beta$ -cells were not observed in the normal pancreas. Unfortunately, the percentage of replicating non- $\beta$ -cells in the capsules could not be reliably quantified, as discussed in METHODS.

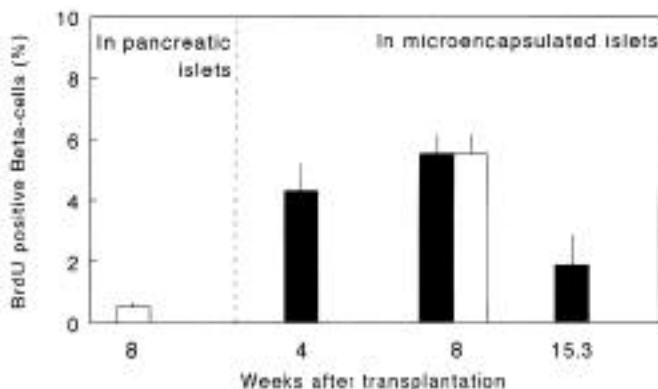


FIG. 5. Percentage of  $\beta$ -cell replication in islets in the normal pancreas and in microencapsulated islets at several time points after implantation. The normal percentage of replicating  $\beta$ -cells was measured in the pancreases of nondiabetic recipients (□) subjected to peritoneal lavage at 8 weeks after implantation. ■, results in animals that were diabetic immediately preceding the implantation. The *n* values are similar to those given in Table 1. Values represent means  $\pm$  SE.

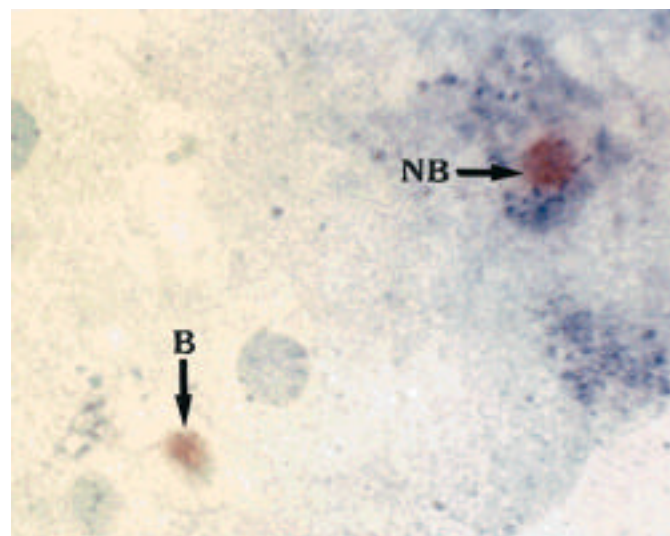


FIG. 6. Replicating islet cells in a microencapsulated islet, 8 weeks after successful allotransplantation in a diabetic rat. NB, replicating non- $\beta$ -cell; B, a not-completely-sliced nucleus of a replicating  $\beta$ -cell. At the time of peritoneal lavage, the animal was still normoglycemic. GMA-embedded section was double stained for the non- $\beta$ -cell hormones (glucagon, pancreatic polypeptide, and somatostatin) and BrdU. Original magnification  $\times 1000$ .

With the exception of a few encapsulated islets smaller than 100  $\mu$ m, all encapsulated islets contained necrotic cells. This necrosis varied from single-cell necrosis to almost complete necrosis of the islets, with only a rim of vital  $\beta$ -cells. Necrosis in the islets was observed to occur mainly centrally and only occasionally peripherally. The percentage of necrotic tissue in the encapsulated islets increased gradually with time (Fig. 7) and was associated with a gradual decrease in volume of the vital  $\beta$ -cell mass. The severity of necrosis was not associated with the state of glycemia in the recipients, since at 8 weeks after implantation we found the percentage of necrosis to be similar in recipients that were normoglycemic and diabetic before implantation (Fig. 7).

Cells positively stained for apoptosis were rarely observed in encapsulated islets. Apoptotic islet cells were absent in

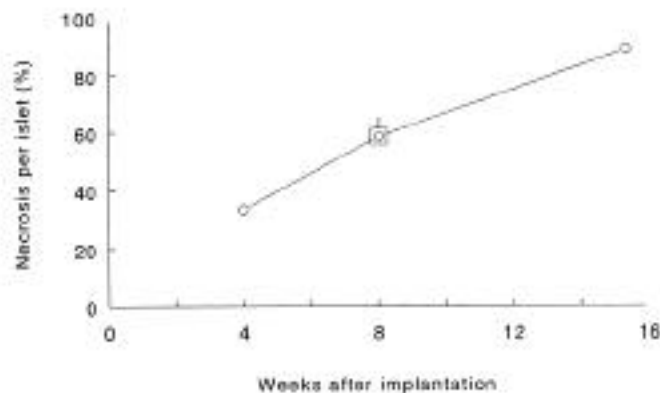


FIG. 7. Mean percentage of necrosis in islets at 4 and 8 weeks after transplantation and at 2 weeks after the recurrence of hyperglycemia. ●, recipients that were diabetic preceding transplantation; □, recipients that were normoglycemic preceding transplantation. The *n* values are similar to those given in Table 1. Values represent means  $\pm$  SE.

most slices and present only in low and insufficient numbers for reliable quantification in the slices with positively stained cells. In contrast to necrotic cells, apoptotic cells were observed not only in the center but also in the periphery of the islets. Since the TDT method only stains cells in the process of apoptosis, and since this process is complete in not more than a few minutes, the TDT method is a measure for the number of apoptotic cells at the moment of explantation of the graft and not a reliable technique for quantification of the contribution of apoptosis to the involution of the graft during the 13.3 weeks of function. Therefore, we determined the number of apoptotic bodies in the encapsulated islets. Because the size of apoptotic bodies does not allow passage over the capsule membrane and apoptotic bodies are therefore likely to accumulate if apoptosis occurs at high frequency, the number of apoptotic bodies in the capsules serves as a measure for the contribution of apoptosis to the involution of the graft in a retrospective fashion. Apoptotic bodies were as rarely observed as apoptotic cells at the three time points tested, however, which suggests that necrosis rather than apoptosis is the major process responsible for islet cell death in the encapsulated islets.

## DISCUSSION

Our present study demonstrates, for the first time, that factors other than fibrotic overgrowth contribute to failure of encapsulated islet grafts. It shows that capsular overgrowth is not progressive, as generally assumed (10,16,35,36), but rather the consequence of a reaction during the first few weeks after implantation against not more than a small percentage of the capsules. As such, the number of islets that escape from this reaction (4  $\mu$ l of islet tissue) is sufficient for maintaining normoglycemia (21,37,38). But a further involution of the graft is brought about by an imbalance in  $\beta$ -cell birth and death in the non-overgrown encapsulated islets, as illustrated by the progression of necrosis in spite of high replication rates of  $\beta$ -cells.

Remarkably, ~40% of the islets are lost in the first 4 weeks after transplantation, although only a small percentage of the capsules are caught in fibrosis. This fibrosis, however, may have caused a higher loss of islets than is to be expected on the basis of the number of observed overgrown capsules. This finding can be explained as follows. Approximately 50% of the capsules of an encapsulated islet graft do not contain an islet (16). The fibrotic reaction is mainly directed against capsules with physical imperfections, which are much more frequent with islet-containing than with empty capsules (16,39), implying that the overgrown capsules are mainly islet-containing rather than empty capsules. Consequently, the loss of islets associated with the 10% of overgrown capsules retrieved with lavage is higher than this 10% and probably closer to 20% of the islets, since the percentage of empty capsules is 50% (16,39). Furthermore, a portion of ~10% of the capsules is not retrieved by peritoneal lavage, as the consequence of tight adherence to the abdominal organs (10,11,35), implying an additional loss that is >10% and, probably, closer to 20% of the transplanted islets. Thus, in total, capsular overgrowth accounts for an expected loss of at least 20% of the initially transplanted islets, and it may approximate 40%, which is the percentage of islets lost in the first 4 weeks as experimentally determined. Our observations, therefore, suggest that in studies addressing the survival of microen-

capsulated islets, the number of retrieved islet-containing capsules should be measured in addition to the total number of retrieved capsules, since the number of retrieved capsules is not necessarily representative for the number of surviving islets.

The percentage of 4–6% of replicating  $\beta$ -cells in the encapsulated islets illustrates a high renewal of insulin-producing cells, since  $\beta$ -cell birth is ~0.6% per day in the normal non-stimulated AO/G-rat pancreas. Such a high renewal rate has been reported before (29–31). Montaña et al. (29,30) and Nacher et al. (31) found 1.26–1.39% of the  $\beta$ -cells to be positive for BrdU after 6 h of labeling, a percentage similar to the fourfold higher rate of 4–6% that we found after 24 h of labeling. These high renewal rates were only observed in grafts composed of small numbers of islets, since these islets are subject to a high metabolic demand (29–31). However, the volume of our grafts was not small but quite large at 4 weeks postimplant—it was 4  $\mu$ l of islet tissue, which corresponds to 40% of the volume of islet tissue in the native rat pancreas, a sufficiently large volume to induce permanent normoglycemia when islet-transplanted as a nonencapsulated islet graft into the peritoneal cavity (38) or to any other commonly used islet transplantation site in the rat (21,37). A second argument in favor of nonmetabolic factors as the cause of the high replication rates is that high numbers of replicating  $\beta$ -cells were also found in nondiabetic recipients, that is, in the absence of any increased metabolic demand.

Our implantation studies in nondiabetic recipients show that the stimulation of replication is caused by local factors in the peritoneal cavity rather than by systemically distributed replication stimuli such as glucose (29,30,40–44), since high numbers of replicating  $\beta$ -cells were only observed in the intraperitoneally implanted encapsulated islets and not in the islets in the pancreas of the same recipient. Our studies also demonstrate that the stimulation of the proliferation of islet cells is nonspecific, since we observed not only high numbers of replicating  $\beta$ -cells but also high numbers of replicating non- $\beta$ -cells. Possible candidates for this local, nonspecific, stimulation of islet-cell replication are cytokines produced by macrophages on the overgrown capsules which diffuse into the non-overgrown capsules (45). Several cytokines produced during inflammation, such as interferon- $\gamma$  and interleukin-1 $\beta$ , are known to stimulate  $\beta$ -cell replication (45,46), either directly or indirectly via stimulation of production of growth factors. Another possibility is that necrotic islet cells release a signal for replication (45). This suggestion is supported by several reports (45,47,48) showing proliferation of islet cells in response to  $\beta$ -cell toxins.

The hyperproliferation warrants some further consideration, since it should be interpreted not only as insufficient to compensate for the increased cell death but also as a detrimental factor in itself. Two mechanisms may be involved. Because  $\beta$ -cells have limited capacity for repetitive replication (49–53), the  $\beta$ -cells may lose their capacity for compensatory renewal of cells after the replication at high frequency during the 13.3 weeks of graft function. This suggestion is corroborated by our observation that  $\beta$ -cells cease to proliferate in response to the elevated glucose levels at the moment of graft failure, which normally is a strong stimulus for replication of islet cells (29). We have observed cell death to be restricted to the  $\beta$ -cells but cell renewal to occur in all types of islet cells. This disturbs the normal ratio of insulin-pro-

ducing and non-insulin-producing endocrine cells, which might interfere with islet function (54,55).

Apoptosis was quantified not only by a labeling technique but also by morphologically identifying cells with characteristics of apoptosis and apoptotic bodies. We recognize that the morphologic approach carries the risk of underestimating the involvement of apoptosis as a consequence of necrosis of apoptotic bodies, that is, secondary necrosis. By combining the labeling and morphologic approach, however, we can safely conclude that necrosis and not apoptosis is the main cause of cell death and failure of the graft, since in the thousands of evaluated capsules we found only a few apoptotic cells, slightly more but still very few apoptotic bodies, no necrotic apoptotic bodies, and large numbers of cells with the characteristics of necrosis.

The observation that the necrosis is mainly located in the central part of the encapsulated islets suggests insufficient nutrition as a major cause of graft failure. This insufficient nutrition is a consequence of the fact that microencapsulated islets stay unattached and freely floating in the peritoneal cavity without any vascularization, which implies that supply of nutrients depends on diffusion from blood to the peritoneal cavity. This diffusion has been shown to be rather slow (56,57) which, together with the low oxygen tension (58–60) and low concentration of essential minerals (61,62) in the peritoneal cavity, predictably interferes with the optimal nutrition of the islets.

One could argue that better nutrition may be obtained by using smaller capsules. However, we have previously shown that islets are usually in the periphery of the capsules (22,39) where nutrition is already optimal and that, consequently, the supply of nutrients will not dramatically improve by reducing the capsule size. This is corroborated by the findings of Sun and colleagues (63,64) that graft survival with smaller capsules is not permanent but limited to several months.

Research efforts should focus on two issues. One is to improve nutrition of the islets to reduce necrosis in the islets. A conceivable approach is to find or to create a transplantation site that, more than the unmodified peritoneal cavity, permits for close contact between the bloodstream and the encapsulated islet tissue. The other is to identify and abolish the causative factors for the enhanced proliferation in the encapsulated islets.

#### ACKNOWLEDGMENTS

This work was supported by a grant of the Diabetes Research Fund of the Netherlands.

Dr. H. van Goor, Department of Pathology, Groningen, the Netherlands, is greatly acknowledged for his helpful suggestions during the set-up of the staining procedures. The monoclonal antibody against BrdU was kindly provided by Prof. Dr. L. de Leij, Department of Clinical Immunology, Groningen, the Netherlands.

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