

## Factors Influencing Functional Survival of Microencapsulated Islet Grafts

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Graft function of encapsulated islets is restricted in spite of the fact that inflammatory responses against capsules are limited to a portion less than 10%. It has been shown that dysfunction is accompanied by a gradual decrease in the glucose-induced insulin response (GIIR), a hyperproliferation of islet cells, and gradual necrosis. Also, limited survival is associated with the presence of macrophages in the overgrowth. In the present study, we investigate whether macrophages are the inducers of dysfunction of encapsulated grafts. Four weeks after successful transplantation of microencapsulated rat allografts we determined the GIIR, the rate of islet cell replication, and islet cell death. Also, we quantified the number of macrophages on the overgrown capsules. This assessment was applied to set up an *in vitro* coculture system of macrophages and encapsulated islets. We retrieved 93 ± 6.2% of the capsules of which 9.2 ± 0.3% was overgrown. The GIIR of the retrieved nonovergrown islets was reduced when compared with freshly encapsulated islets. The replication rate of the retrieved islet cells was eightfold higher than in the normal pancreas. Apoptosis was rarely observed but 37 ± 4% of the total islet surface was composed of necrosis. We found a mean of 1542 ± 217 macrophages per capsule. Coculture of 1500 NR8383 macrophages per encapsulated islets induced a substantial reduction in GIIR but a decrease instead of increase in replication. Necrosis was restricted to 13 ± 1.3% of the islet cells and was not increased by the presence of macrophages. Our observations indicate that we should focus on reduction of macrophage activation and on improving the nutrition of encapsulated islets to prevent islet cell death.

**Key words:** Islets; Macrophages; Alginate; Poly-L-lysine; Encapsulation; Immunoisolation

### INTRODUCTION

Encapsulation in alginate–poly-L-lysine (PLL)-based microcapsules (22) is a commonly applied procedure for immunoprotection of pancreatic islets. During recent years, important advances have been made with this technology. The introduction of pure and new types of alginates (7,18,28) and methods for testing the integrity of the capsules has been associated with a substantial reduction of fibrotic overgrowth from all capsules to a portion of 2–10% of the capsules (7,13). This improvement of biocompatibility is beneficial for functional graft survival, because normoglycemia was observed during 2 to 6 months in streptozotocin diabetic recipients of microencapsulated islet allo- and isografts (7,23,27).

Although this illustrated the principle applicability of the alginate encapsulation technique, a fundamental barrier needed to be overcome because graft survival varied considerably in our studies from 2 to 6 months and was never permanent (5,7,11). Obviously, processes other

than fibrotic overgrowth with subsequent necrosis of the islets contribute to failure of the encapsulated islet grafts. These are islet-related factors such as limited life span of islets (1,15) but also indirect effects of the tissue response against the encapsulated islets, as will be discussed in the next sections.

The cellular overgrowth on the small portion of 2–10% of the capsules is mainly composed of ED-1 and ED-2 macrophages (8). In a previous study we suggested that these macrophages contribute to cessation of the graft not only by overgrowth and subsequent necrosis of the islets but also by secretion of cytokines with potential deleterious effects on the islets in 90–98% of nonovergrown encapsulated islets.

The present study was undertaken to study the mechanism and quantify the effect of macrophages in the 2–10% overgrowth on the nonovergrown encapsulated islets. To this end, we studied transplanted microencapsulated islets at 4 weeks after implantation. At the time of peritoneal lavage, encapsulated islets were tested for

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their *ex vivo* insulin response to glucose, the rate of islet cell proliferation, and the percentage of nonviable tissue in the islets because we have observed disturbed patterns for these parameters in previous studies (13). To investigate whether these assessments are correlated with the presence of macrophages in the immediate vicinity of encapsulated islets, we applied a coculture system of encapsulated islets with macrophages to mimic the *in vivo* situation.

## MATERIALS AND METHODS

### *Design of the Study*

Alginate-PLL microcapsules were implanted in the peritoneal cavity (i.e., the usual transplantation site for an encapsulated islet graft) of AO rats. Only highly purified alginates were applied to exclude that contaminating components were the cause of an inflammatory response. The capsules were inspected before and after implantation to confirm that the majority of the capsules were intact. The capsules were retrieved at 4 weeks postimplant by peritoneal lavage.

At the time of peritoneal lavage the encapsulated islets were divided in two portions. The first portion was applied to determine the *ex vivo* insulin response to glucose, the rate of islet cell proliferation, and the percentage of nonviable tissue in the islets. The second portion was used for determination of the percentage of overgrowth of the encapsulated graft and for quantification of the number of macrophages in the capsular overgrowth.

The total volume of the graft recovered by peritoneal lavage was related to the initial volume of the transplant. The number of recovered capsules with cellular overgrowth was assessed by microscopy of histological sections. This fraction was expressed as the percentage of the total number of recovered capsules.

The number of ED-1 and ED-2 positive macrophages in the overgrown capsules was quantified by microscopy of histological sections of the graft. The counted number of macrophages was related to the percentage of overgrown capsules in the graft. The number of macrophages was expressed as the quantity of macrophages per capsule. These assessments were applied to culture rat macrophages (NR8383) with 100 encapsulated Lewis rat islets. After 48 h of coculture with macrophages, encapsulated islets were harvested and tested for their ability to respond to a glucose load with an insulin response and processed for histological determination of the number of replicating islet cells, and the percentage of nonviable tissue. Cocultures of LPS-activated rat macrophages with encapsulated islets were applied to assess the protective effect of capsules for macrophage-derived cytokines. Cultures of encapsulated Lewis rat islets served as control.

### *Animals and Induction of Diabetes*

Male inbred Albino Oxford (AO/G) and Lewis rats were obtained from Harlan (Harlan CPB, Zeist, The Netherlands) or the Central Animal Laboratory of Groningen. NHI guidelines for the care and use of laboratory animals were observed.

Male inbred Lewis rats weighing 300–350 g served as donors. Male inbred AO/G rats weighing 290–320 g were used as recipients of encapsulated islet grafts. Diabetes was induced by injection of 75–90 mg/kg of streptozotocin (Zanosar, Upjohn Co., Kalamazoo, MI) via the tail vein. Glucose concentration in blood was determined with glucose test tapes (Reflolux, Boehringer Mannheim, FRG). Only animals with severe weight loss, polyuria, polydipsia, and blood glucose levels exceeding 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.

### *Islet Isolation*

Islets were isolated as previously described (32). 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 (KRH) supplemented with 25 mmol/L HEPES buffer 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 two-stage incubation of 20 min at 37°C with successively 1.0 and 0.7 mg/ml collagenase (Sigma type XI, Sigma, St. Louis, MO). Islets were separated from exocrine tissue by centrifugation over a discontinuous dextran gradient (31) and further purified by handpicking.

The total islet volume was determined by measuring the diameters (30) of islets in a 4% aliquot of the islet suspension. 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 a total of 3500–4200 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 nontreated petri dishes (Greiner, Alphen a/d Rijn, The Netherlands) in portions of 100 islets per 25 cm<sup>2</sup> for 19–44 h in RPMI-1640 [containing 10% fetal calf serum (FCS, Gibco, Breda, The Netherlands), 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. Graft endocrine volume was at least 10 µl, which is approximately equal to the endocrine volume of the normal rat pancreas (31).

#### *Microencapsulation*

After culture, islets were washed three to five times with RPMI containing 10% FCS and were subsequently suspended in sterile filtered (0.2 µm filtration) 3–3.3% purified alginate solution. The alginate solution was converted into droplets using an air-driven droplet generator as previously described (6,33). PLL–alginate encapsulation was performed as described elsewhere (17). Briefly, the alginate droplets were transformed to alginate beads by gelling in a 100 mM CaCl<sub>2</sub> (10 mM HEPES, 2 mM KCl) solution for at least 5 min. Subsequently, the Ca–alginate beads were suspended for 1 min in KRH containing 2.5 mmol/L CaCl<sub>2</sub>. A PLL membrane was formed by suspending the alginate beads in 0.1% PLL solution for 10 min (PLL-HCl, MW 22,000, Sigma). Nonbound PLL was removed by three successive washings during 3 min with Ca<sup>2+</sup>-free KRH containing 135 mM NaCl. The outer alginate layer was subsequently applied by 5-min incubation in 10 times diluted alginate solution. The capsules had a diameter of 600–700 µm as measured with a dissection microscope (Bausch and Lomb BVB125) equipped with an ocular micrometer with an accuracy of 10 µm. The total graft volume was determined in a measure cylinder, with an accuracy of 0.05 ml.

#### *Transplantation and Retrieval of Microencapsulated Islets*

Under halothane anesthesia, the microencapsulated islets were injected into the peritoneal cavity with a 16-gauge cannula via a small incision (3 mm) in the linea alba. The abdomen was closed with a two-layer suture. The implanted volumes varied between 2.0 and 2.5 ml. Blood glucose was determined at regular time intervals in blood sampled from the tail vein. The glucose concentration was determined with glucose test tapes (Reflolux). Recipients with a blood glucose level less than 8.4 mmol/L were considered normoglycemic.

Microcapsules were retrieved at 4 weeks after transplantation. Microcapsules were mostly freely floating and nonadherent, but occasionally we found some capsules adhering to the surface of intra-abdominal organs. First, nonadherent microcapsules were retrieved by peritoneal lavage, performed by infusing 20 ml KRH through a 3-cm midline incision into the peritoneal cavity and subsequent flushing with additional KRH for two or three times above a 50-ml centrifuge tube. The cap-

sules were immediately fixed after lavage to avoid inappropriate activation of the adherent cells (vide infra). From this tube, the microcapsules were brought into a measure cylinder to assess the retrieved volumes with an accuracy of 0.05 ml as described above. Also, we confirmed that the diameter of the capsules had not changed during implantation and subsequent fixation because a change in diameter of the capsules could interfere with adequate measurement of the retrieved graft volume (13). Subsequently, the microcapsules adhering to the surface of abdominal organs were excised and processed for histology.

#### *Cell Cultures and Co-incubation*

Before starting the in vitro experiments we selected an appropriate macrophage source for mimicking the in vivo situation. To this end, we first tested the feasibility of using residential macrophages either from overgrown capsules, from peripheral blood, or (protease–peptone-elicited) peritoneal macrophages (2). This was done by comparing the number of iNOS-positive macrophages obtained from the in vitro system with those collected in vivo on capsules directly fixed after being flushed from the peritoneal cavity. Unfortunately, none of the sources were adequate for application in the in vitro system because isolation and processing of the macrophages for in vitro application were associated with strong and inappropriate activation of the cells as evidenced by iNOS expression in 80–95% of the macrophages, while it was always less than 50% of the macrophages directly fixed after flushing from the peritoneal cavity. Another reason prohibiting the use of the above-mentioned macrophages as a cell source was that it was not applicable for long-term cultures because a substantial number of the macrophages died within hours after starting the incubations at 37°C. Therefore, in our subsequent approach we concentrated on finding an appropriate macrophage cell line instead of resident macrophages.

In total we have tested the adequacy of eight different cell lines. In these tests we not only studied and compared iNOS expression but also the ability of the cell line to secrete IL-1β and TNF-α in a dose-dependent fashion upon activation with lipopolysaccharide (LPS, *E. coli*, Sigma). Only the Sprague-Dawley macrophage cell line NR8383 (ATCC, Manassas, VA) (19) proved to be adequate for application in our test system because processing for culture was not associated with increased expression of iNOS and it was one of the few cell lines that was found to secrete IL-1β and TNF-α in a dose-dependent fashion upon activation with LPS.

Before application in the experiments, the NR8383 macrophage cell line was cultured in 60-ml culture flasks containing RPMI-1640 with 10% fetal calf serum,

8.3 mmol/L glucose, 10 mmol/L HEPES, and 1% penicillin/streptomycin. The cultures were kept at 37°C in humidified air containing 5% CO<sub>2</sub>.

In the co-incubation experiment four separate samples of 100 encapsulated islets were incubated with an appropriate quantity of macrophages (see Results section) in 12-well plates and subsequently cultured for 48 h in RPMI-1640 with 10% fetal calf serum, 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>. As a negative control on macrophage activation we used the results of cultures of encapsulated islets alone without macrophages. NR8383 macrophages activated with 1 µg/ml LPS served as positive controls. This amount of LPS maximally stimulates cytokine production (8). Islets were harvested and processed for measurement of insulin secretion during glucose challenge or processed for glycol methacrylate (GMA) embedding as described below.

#### *BrdU Labeling of Islets*

To label replicating cells *in vivo*, a bromo-2-deoxyuridine (BrdU) tablet (Boehringer Mannheim) was implanted subcutaneously via a small incision in the skin of the neck at exactly 6 h before peritoneal lavage.

For BrdU labeling of encapsulated islet cells *in vitro*, islets were incubated for 6 h in culture medium containing 10 µM BrdU. BrdU labeling was always performed in culture medium containing 15 mM glucose, a concentration that stimulates islet cell replication (25).

#### *Insulin Secretion During Glucose Challenge*

Directly after peritoneal lavage, microencapsulated islets were tested *ex vivo* 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 µm. The encapsulated islets were preincubated for 45 min in 2 ml Krebs-Ringer-bicarbonate (KRB), gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, containing 0.25% BSA and 2.75 mM glucose. The *ex vivo* insulin secretion was then assessed by three consecutive incubations of (i) 45 min in 2.75 mM glucose in KRB, (ii) 45 min in 16.5 mM glucose in KRB, and (iii) 45 min in 2.75 mM glucose in KRB. At the end of each incubation, the incubation media were removed and frozen for insulin determination by a radioimmunoassay for insulin (Linco, St. Charles, MO) using rat insulin as a standard. Groups of 25 freshly isolated and encapsulated islets served as control. The insulin secretory responses were expressed as picogram of insulin L<sup>-1</sup> 25 islets<sup>-1</sup> 45 min<sup>-1</sup>.

Islets obtained from culture experiments were tested according to the same procedure with one exception. We applied four separate samples of 10 islets each instead

of samples of 25 islets each. Consequently, the insulin secretory responses of islets from *in vitro* culture experiments were expressed as nanogram of insulin ml<sup>-1</sup> 10 islets<sup>-1</sup> 45 min<sup>-1</sup>.

#### *Histology and Assessment of Capsular Overgrowth*

Nonadherent capsules recovered by peritoneal lavage were always divided in three portions. One portion was fixed in precooled 2% paraformaldehyde, buffered with 0.05 M phosphate, and processed for GMA embedding (4), the second portion was frozen in precooled iso-propan (5), and the third portion was fixed in Bouin's solution for paraffin processing (16).

GMA sections were prepared at 2 µm and used for the following assessments. GMA sections stained with Romanovsky-Giemsa were used to assess the degree of capsular overgrowth, which was quantified by expressing the number of recovered capsules with overgrowth as the percentage of the total number of recovered capsules for each individual animal. The same sections were used for assessing the percentage of necrotic tissue in the islets as previously described (13). Briefly, necrosis in the retrieved islets was assessed by identifying cells or groups of cells in the islets with the morphological 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. 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. Also, GMA sections of nonadherent capsules were processed for identification of BrdU-positive cells in the β-cell fraction of the islets. To this end, we applied a double staining protocol for the non-β-cell hormones and BrdU as previously described (13). We choose to stain the non-β-cells in the islets in combination with BrdU instead of β-cells and BrdU because in the grafts we often found degranulated islet cells that would be missed as β-cells when the latter technique was applied. The adequacy of this approach has been shown in a previous study (13).

The sections were incubated with a cocktail of the following antibodies: rabbit anti-glucagon (DAKO, Santa Barbara, CA), rabbit anti-human somatostatin (DAKO), and rabbit anti-human pancreatic polypeptide (DAKO). After several washings, sections were incubated with a goat anti-rabbit, alkaline phosphatase conjugated second step reagent. Phosphatase activity was demonstrated by applying Naphtol 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. 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. All cells in the sections were counted by an investigator. At least 600 cells were counted per graft. The number of BrdU-positive  $\beta$ -cells was expressed as the percentage of the total number of counted  $\beta$ -cells.

The second portion of the recovered capsules was sectioned at 5  $\mu$ m and processed for immunohistochemical staining as previously described (5). The monoclonal antibodies used were: a combination of ED-1 and ED-2 against monocytes and macrophages and subset of macrophages (14), HIS-40 against IgM bearing B-lymphocytes (3), HIS-48 against neutrophilic granulocytes (29), R73 against CD3<sup>+</sup> bearing T-lymphocytes (20), and ASIALO-GM1 directed to natural killer (NK) cells (26). After several washing steps the sections were incubated with appropriate horseradish peroxidase-conjugated second step reagents. Peroxidase activity was demonstrated by applying 3,3'-diaminobenzidine tetra HCl containing hydrogen peroxide. In control sections we used PBS instead of the first stage monoclonal antibody. This second portion was not only used to study the composition of the overgrowth but also to assess the number of ED-1 and ED-2-positive macrophages in the capsular overgrowth. To this end, we counted the macrophages in each graft on at least 100 sectioned overgrown capsules. Then we calculated for each graft the total number of cells on the overgrown capsules, considering the capsules to be perfect spheres. The cell number was expressed as a mean number of cells per recovered capsules for each individual animal.

The third portion of nonadherent capsules was 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 deoxynucleotidyl 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 (i.e., 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.

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

BrdU-labeled islets obtained from in vitro experi-

ments were fixed in precooled 2% paraformaldehyde, buffered with 0.05 M phosphate, and processed for GMA embedding. Sections were prepared at 5  $\mu$ m and processed for immunocytochemical staining of BrdU-positive islet cells with staining for non- $\beta$ -cell hormones. Quantification of islet cell replication was performed in the same way as specified above for  $\beta$ -cell replication. At least 500 islet cells were counted per experiment.

Pancreas biopsies were fixed in Bouin's solution for paraffin processing, sectioned at 5  $\mu$ m, and processed for immunocytochemistry as described above for determination of the number of BrdU-positive cells or for identification of apoptotic  $\beta$ -cells.

#### Statistical Analysis

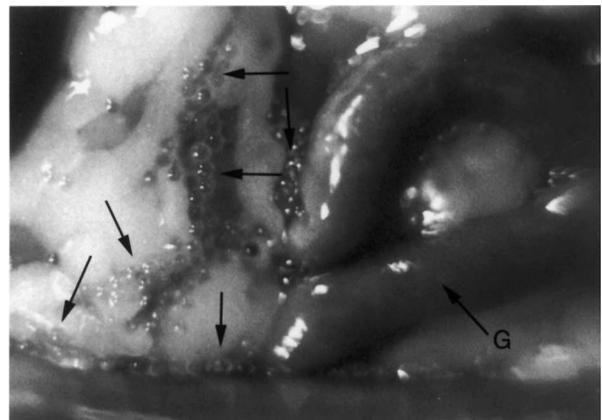
Results are expressed as mean  $\pm$  SEM. Statistical comparisons were made with the Mann-Whitney *U* test. A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

### Composition and Degree of Cellular Overgrowth of the Grafts

All five diabetic AO recipients of encapsulated Lewis rat islets became normoglycemic within 5 days after implantation and remained normoglycemic for the study period of 4 weeks. Encapsulated grafts were retrieved by peritoneal lavage at 4 weeks after implantation.

The majority of the capsules of 93  $\pm$  6.2% were freely floating in the abdominal cavity without adhesion to the abdominal organs (Fig. 1). Occasionally, we found a portion of the capsules adhering to the omentum or liver capsule. All capsules adhering to the abdominal



**Figure 1.** Free-floating alginate-PLL encapsulated islets in the peritoneal cavity of cured AO rats 1 month after implantation. The arrows indicate free-floating capsules in between the abdominal organs. G indicates a gut.

organs were found to be overgrown by fibrotic tissue and occasionally by some macrophages. Of the 93  $\pm$  6.2% capsules retrieved by peritoneal lavage, the mean percentage of capsules with overgrowth was  $9.2 \pm 0.3\%$  and varied between 1% and 14%. The composition of the overgrowth on the  $9.2 \pm 0.3\%$  of free-floating capsules was different from that on capsules adhering to the abdominal organs. The free-floating capsules were surrounded by some fibrotic tissue but the vast majority of cells were ED-1- and ED-2-positive macrophages. NK cells, granulocytes, CD3<sup>+</sup>-bearing T-lymphocytes, or B-cells were never observed in the histological slices.

The number of ED-1- and ED-2-positive macrophages was quantified in histological slices of free-floating capsules. The total number of macrophages varied between 10,000 and 24,000 per overgrown capsule. From these assessments follows that with a 9.2% overgrowth rate the quantity of macrophages per capsule amounts to  $1542 \pm 217$ .

#### *Function, Replication, and Cell Death in Explanted Encapsulated Islet Grafts*

As shown in Figure 2, the explanted microencapsulated islets always responded to glucose stimulation. However, the magnitude of the response was reduced ( $p < 0.05$ ) when compared with freshly encapsulated islets. Also, we found, as in our previous study on explanted encapsulated islets (13), an incomplete return to basal insulin secretion ( $p < 0.05$ ) during the second incubation in low glucose.

In islets collected by peritoneal lavage we specifically determined the percentage of replicating  $\beta$ -cells.

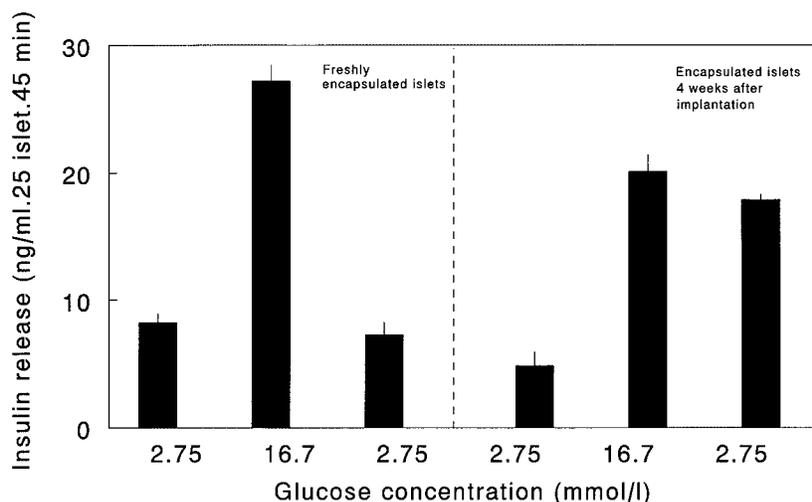
The normal percentage of  $\beta$ -cell replication was determined in the pancreas of age-matched nondiabetic controls. As shown in Figure 3,  $\beta$ -cell replication in normoglycemic recipients was eightfold higher in encapsulated islets than in islets in the normal pancreas.

With the exception of a few encapsulated islets smaller than 100  $\mu\text{m}$ , 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 islet cells. Necrosis in the islets was observed to occur almost exclusively in the center and only occasionally in the periphery. In total  $37 \pm 4\%$  of the surface of the islet was composed of necrotic cells.

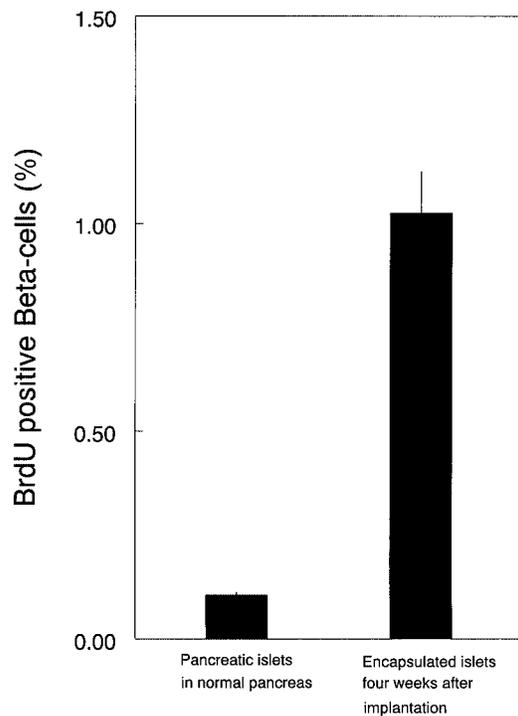
Cells positively stained for apoptosis were rarely observed in encapsulated islets. Apoptotic cells were absent in most grafts and present only in low and insufficient numbers for reliable quantification in the slices with positively stained cells. The observation that apoptotic cells are rarely present in encapsulated islets confirms our previous observations (13) that necrosis is the major process responsible for islet cell death in the encapsulated islets. In view of these results we focused our subsequent *in vitro* studies on proliferation and necrosis only and abandoned quantification of apoptosis in microencapsulated islets.

#### *The Effect of Rat Macrophages on Function, Replication, and Cell Death in Encapsulated Islets In Vitro*

We questioned whether the reduced insulin responses, the hyperproliferation, and the high number of necrotic cells in the implanted encapsulated islets are caused by



**Figure 2.** Ex vivo glucose-induced insulin response of Lewis rat islets at 4 weeks after implantation (right panel) and of freshly encapsulated, control islets (left panel). Values represent mean  $\pm$  SEM of five experiments.



**Figure 3.** The percentage of replicating  $\beta$ -cells in islets in the normal pancreas and in microencapsulated islets at 4 weeks after implantation. Values represent mean  $\pm$  SEM of five experiments.

bioactive molecules secreted by the macrophages on the  $9.2 \pm 0.3\%$  of overgrown capsules. To answer this question we designed a coculture system with 1500 macrophages per islet-containing capsule (i.e., the mean number of macrophages per capsule, *vide supra*), to mimic the *in vivo* situation.

As shown in Figure 4, co-incubation with macrophages had a clear-cut effect on the glucose-induced insulin response of encapsulated islets. We observed a pronounced reduction of the basal insulin secretion ( $p < 0.05$ ) and the glucose-stimulated insulin secretion ( $p < 0.02$ ) when compared with controls. Also, we observed an incomplete return to basal insulin levels after glucose challenge. This effect of macrophages on insulin secretion was even more pronounced in co-incubations of encapsulated islets with LPS-activated macrophages, because islets in capsules had completely lost their ability to respond to a glucose load when LPS-activated macrophages were in their direct vicinity.

Co-incubation of encapsulated islets with macrophages did not bring about a hyperproliferation of the encapsulated islets cells (Fig. 3) as observed in explanted encapsulated islets but rather a sharp decrease in the proliferation rates (Fig. 5). Co-incubation of islets with macrophages caused a threefold decrease in the replica-

tion rate of  $\beta$ -cells. This effect on proliferation of islet cells was even more noticeable in co-incubations of encapsulated islets and LPS-activated macrophages, because  $\beta$ -cells in capsules had completely lost their ability to replicate.

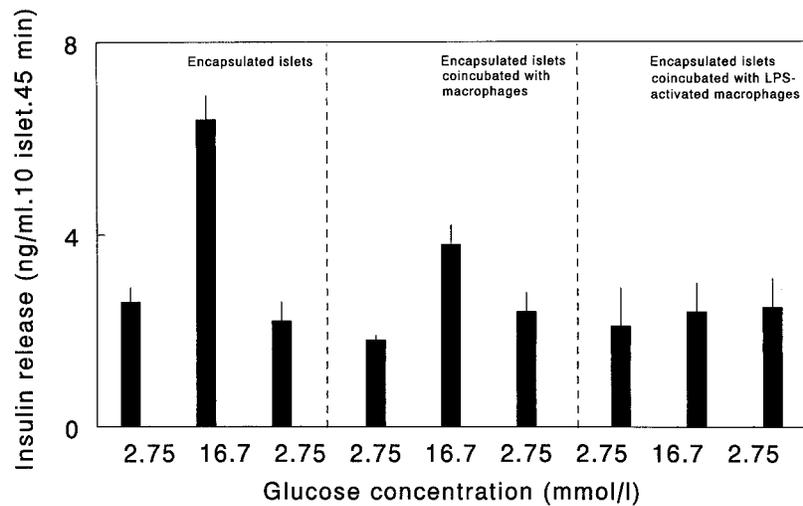
In the absence of macrophages, encapsulated islets were composed of  $13 \pm 1.3\%$  ( $n = 5$ ) of necrotic cells. This necrosis varied from single-cell necrosis to almost complete necrosis of the center of the islets. Necrosis was observed to occur mainly centrally and only occasionally peripherally. The severity of necrosis was not associated with the presence of macrophages in the vicinity of the capsules because the mean percentage of necrosis was similar if not identical in encapsulated islets cultured with macrophages or with LPS-activated macrophages. Also, the severity of necrosis was not increased by the presence of the capsule because naked, nonencapsulated islets showed  $11 \pm 3.2\%$  of necrotic cells ( $n = 5$ ) in the islets, a percentage similar to the percentage of necrosis in encapsulated islets.

## DISCUSSION

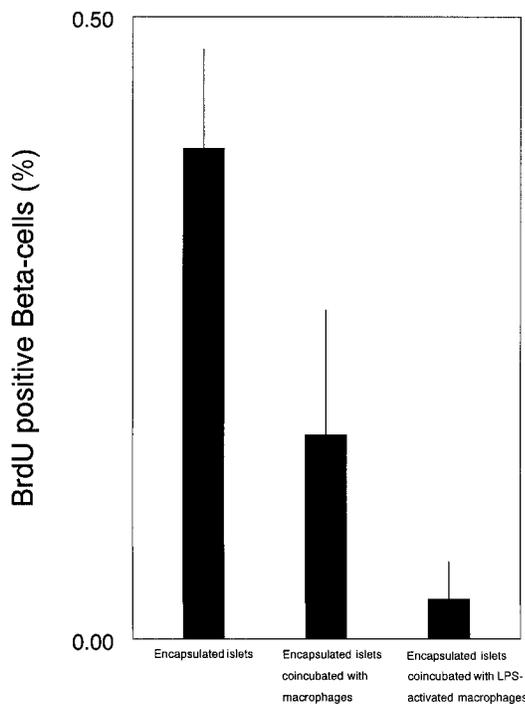
It is generally assumed that the loss of islets associated with overgrowth of 2–10% of the encapsulated islets has no consequences for the function of the remaining 90–98% nonovergrown part of the graft. In the present study we closely mimicked the *in vivo* situation to evaluate the validity of this assumption. We show that macrophages on the 2–10% of overgrown capsules can influence the functional survival of the graft not only by overgrowth and subsequent necrosis of the islets but also by release of deleterious bioactive molecules that diffuse into the 90–98% nonovergrown capsules. These macrophage-derived molecules interfere with the efficacy of the transplanted islets, as illustrated by our observation that islets co-incubated with macrophages show a disturbed insulin response to a glucose load.

The observation that explanted encapsulated islets show an incomplete return to basal insulin secretion after glucose challenge confirms our previous study on function of encapsulated islets (13). A similar profile of incomplete return to basal insulin secretion after glucose challenge was observed in our present *in vitro* study when encapsulated islets were cocultured with macrophages. This suggests that macrophage-derived bioactive factors are responsible for the observed incomplete return to basal insulin levels.

Our observation that necrosis and not apoptosis of islet cells is the main cause of cell death and failure of the graft corroborates previous findings (13). The necrosis was mainly located in the central part of the encapsulated islets, which suggests that insufficient nutrition is the major cause of graft failure. This necrosis was more severe in encapsulated islets retrieved from the perito-



**Figure 4.** Insulin response after glucose challenge of encapsulated islets after 48 h of culture, of encapsulated islets cocultured with macrophages for 48 h, and of encapsulated islets cocultured with LPS-stimulated macrophages for 48 h. Values represent mean  $\pm$  SEM of seven experiments.



**Figure 5.** The percentage of replicating  $\beta$ -cells in islets in encapsulated islets after 48 h of culture, of encapsulated islets cocultured with macrophages for 48 h, and of encapsulated islets cocultured with LPS-stimulated macrophages for 48 h. Values represent mean  $\pm$  SEM of seven experiments.

neal cavity than in those harvested in vitro. Plausibly, this is caused by the less favorable in vivo situation in which factors such as oxygen tension are lower than in vitro (11). A new and surprising observation is that the necrosis was not influenced by the presence of a capsule around the islets because the quantity of necrotic cells was similar in free and encapsulated cultured islets. Obviously, the capsule as such does not interfere with diffusion of essential nutrients into the islets. The necrosis is therefore caused by too slow diffusion of nutrients through the outer cells layers of the islets.

Our present data on hyperproliferation of islet cells confirms previous observations that encapsulated islet cells show an extreme increase in proliferation rate after transplantation (13). The hyperproliferation of islet cells in vivo warrants some further consideration, because the hyperproliferation should not be interpreted as insufficient to compensate for the increased cell death but rather as a detrimental factor in itself. At least two mechanisms may be involved, basically by interfering with the islet composition. Because nonendocrine islet cells like fibroblasts have high replication rates, the nonendocrine cell mass will grow faster than the  $\beta$ -cell mass. This occurs predominantly in the capsule periphery, because there the concentration of nutrients is highest. The nutrition in the core of the capsule becomes increasingly insufficient, with ischemia of  $\beta$ -cells and subsequent necrosis as a consequence. We have observed cell death to be restricted to the  $\beta$ -cells but cell renewal to occur in all types of islet cells (13). This disturbs the normal ratio of insulin-producing and non-

insulin-producing endocrine cells, a disturbance known to interfere with islet function (21,24).

The hyperproliferation *in vivo* is not caused by macrophage-derived bioactive factors as illustrated by our *in vitro* observations that coculture of encapsulated islets with macrophages had a suppressive rather than a supportive effect on the replication of  $\beta$ -cells. One may suggest that protracted and prolonged exposure to cytokines *in vivo* instead of short exposure *in vitro* elicits the production of growth factors in islets with stimulatory instead of inhibitory effects on the replicative capacity of islet cells. Although we cannot completely exclude this phenomenon, a more plausible explanation is that other cells types in the vicinity of the macrophages such as epithelial cells and fibroblasts in the abdominal organs secrete trophic factors in response to signals released by the macrophages and islets on and in the capsules.

One could argue that better survival and function of encapsulated islets may be obtained if capsules are applied with a higher degree of biocompatibility. However, in a previous study we have shown that the cellular overgrowth of the capsules is caused by defects on a small portion of the capsules (5,12) and not by systemic, chemical insufficiencies. These small defects cannot be prevented because encapsulation of islets and transplantation protocols are always associated with shear forces that have a damaging effect on a portion of the capsules. Therefore, overgrowth of some capsules has to be accepted as a natural artifact of the technology.

Research efforts should focus on two issues. One is to improve nutrition of the islets to reduce necrosis of the islets. An approach is to find or create a transplantation site that, more than the unmodified peritoneal cavity, permits close contact between the blood stream and the encapsulated tissue (9). The other is to delete the effects of macrophages on the islet graft. A conceivable strategy is to focus on designing immunosuppressive treatment protocols inhibiting macrophage activation in the immediate posttransplant period. These protocols can be temporary rather than permanent because we found in a previous study that the cellular infiltrate on overgrown capsules was not a permanent but only a temporary phenomenon because after 2 months in the peritoneal cavity only fibroblasts surrounding the capsules were observed (7,10).

## REFERENCES

- Adelman, R.; Saul, R. L.; Ames, B. N. Oxidative damage to DNA: Relation to species metabolic rate and life span. *Proc. Natl. Acad. Sci. USA* 85:2706–2708; 1988.
- Albright, J. W.; Jiang, D.; Albright, J. F. Innate control of the early course of infection in mice inoculated with *Trypanosoma muscili*. *Cell Immunol.* 176:146–152; 1997.
- Deenen, G. J.; Hunt, S. V.; Opstelten, D. A stathmokinetic study of B lymphocytopoiesis in rat bone marrow: Proliferation of cells containing cytoplasmic mu-chains, terminal deoxynucleotidyl transferase and carrying HIS24 antigen. *J. Immunol.* 139:702–710; 1987.
- De Haan, B. J.; van Goor, H.; De Vos, P. Processing of immunoisolated pancreatic islets: Implications for histological analyses of hydrated tissue. *Biotechniques* 32:612–619; 2002.
- De Vos, P.; De Haan, B. J.; Pater, J.; Van Schilfgaarde, R. Association between capsule diameter, adequacy of encapsulation, and survival of microencapsulated rat islet allografts. *Transplantation* 62:893–899; 1996.
- De Vos, P.; De Haan, B. J.; Van Schilfgaarde, R. Upscaling the production of encapsulated islets. *Biomaterials* 18:1085–1090; 1997.
- De Vos, P.; De Haan, B. J.; Wolters, G. H. J.; Strubbe, J. H.; Van Schilfgaarde, R. Improved biocompatibility but limited graft survival after purification of alginate for microencapsulation of pancreatic islets. *Diabetologia* 40:262–270; 1997.
- De Vos, P.; Smedema, I.; van Goor, H.; et al. Association between macrophages activation and function of microencapsulated islets. *Diabetologia*; 2003.
- De Vos, P.; Tatarkiewicz, K. Considerations for successful transplantation of encapsulated pancreatic islets. *Diabetologia* 45:159–173; 2002.
- De Vos, P.; Van Hoogmoed, C. G.; De Haan, B. J.; Busscher, H. J. Tissue responses against immunisolating alginate-PLL capsules in the immediate posttransplant period. *J. Biomed. Mater. Res.* 62:430–437; 2002.
- De Vos, P.; Van Hoogmoed, C. G.; van Zanten, J.; Netter, S.; Strubbe, J. H.; Busscher, H. J. Long-term biocompatibility, chemistry, and function of microencapsulated pancreatic islets. *Biomaterials* 24:305–312; 2003.
- De Vos, P.; Van Schilfgaarde, R. Biocompatibility issues. In: Kührtreiber, W. M.; Lanza, R. P.; Chick, W. L., eds. *Cell encapsulation technology and therapeutics*. Boston: Birkhauser; 1999:63.
- De Vos, P.; van Straaten, J. F.; Nieuwenhuizen, A. G.; de Groot, M.; Ploeg, R. J.; De Haan, B. J.; Van Schilfgaarde, R. Why do microencapsulated islet grafts fail in the absence of fibrotic overgrowth? *Diabetes* 48:1381–1388; 1999.
- Dijkstra, C. D.; Dopp, E. A.; Joling, P.; Kraal, G. The heterogeneity of mononuclear phagocytes in lymphoid organs: Distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology* 54:589–599; 1985.
- Finegood, D. T.; Scaglia, L.; Bonner-Weir, S. Dynamics of  $\beta$ -cell mass in the growing rat pancreas: Estimation with a simple mathematical model. *Diabetes* 44:249–256; 1995.
- Fritschy, W. M.; De Vos, P.; Groen, H.; et al. The capsular overgrowth on microencapsulated pancreatic islet grafts in streptozotocin and autoimmune diabetic rats. *Transpl. Int.* 7:264–271; 1994.
- Fritschy, W. M.; Wolters, G. H.; Van Schilfgaarde, R. Effect of alginate-polylysine-alginate microencapsulation on *in vitro* insulin release from rat pancreatic islets. *Diabetes* 40:37–43; 1991.
- Hasse, C.; Zielke, A.; Klock, G.; et al. Amitogenic alginates: Key to first clinical application of microencapsulation technology. *World J. Surg.* 22:659–665; 1998.
- Helmke, R. J.; Boyd, R. L.; German, V. F.; Mangos, J. A. From growth factor dependence to growth factor respon-

- siveness: The genesis of an alveolar macrophage cell line. *In Vitro Cell Dev. Biol.* 23:567–574; 1987.
20. Huning, T.; Wallny, H. J.; Hartly, J.; Lawetsky, A.; Tiefertaler, G. A monoclonal antibody to a constant region of the rat TCR that induces T-cell activation. *J. Exp. Med.* 169:73–78; 1989.
  21. Keymeulen, B.; Anselmo, J.; Pipeleers, D. Length of metabolic normalization after rat islet cell transplantation depends on endocrine cell composition of graft and on donor age. *Diabetologia* 40:1152–1158; 1997.
  22. Lim, F.; Sun, A. M. Microencapsulated islets as bioartificial endocrine pancreas. *Science* 210:908–910; 1980.
  23. O’Shea, G. M.; Goosen, M. F. A.; Sun, A. M. Prolonged survival of transplanted islets of Langerhans encapsulated in a biocompatible membrane. *Biochim. Biophys. Acta* 804:133–136; 1984.
  24. Pipeleers, D.; In’t Veld, P.; Maes, E.; Van de Winkel, M. Glucose-induced insulin release depends on functional cooperation between islet cells. *Proc. Natl. Acad. Sci. USA* 79:7322–7325; 1982.
  25. Popiela, H.; Moore, W. Proliferation of immunocytochemically identified islet beta cells in culture: Effect of growth factors, hormones, and nutrients. *Pancreas* 4:244–248; 1989.
  26. Reynolds, C. W.; Sharrow, S. O.; Ortaldo, J. R.; Heberman, R. B. Natural killer activity in the rat. II. Analysis of surface antigens on LGL by flow cytometry. *J. Immunol.* 127:2204–2205; 1981.
  27. Sun, Y. L.; Ma, X. J.; Zhou, D. B.; Vacek, I.; Sun, A. M. Normalization of diabetes in spontaneously diabetic cynomolgus monkeys by xenografts of microencapsulated porcine islets without immunosuppression. *J. Clin. Invest.* 98:1417–1422; 1996.
  28. Thu, B.; Bruheim, P.; Espevik, T.; Smidrod, O.; Soon-Shiong, P.; Skjak-Braek, G. Alginate polycation microcapsules. I. Interaction between alginate and polycation. *Biomaterials* 17:1031–1040; 1996.
  29. van Goor, H.; Fidler, V.; Wening, J. J.; Grond, J. Determinants of focal and segmental glomerulosclerosis in the rat after renal ablation. *Lab. Invest.* 64:754–765; 1991.
  30. van Suylichem, P. T.; Strubbe, J. H.; Houwing, H.; Wolters, G. H.; Van Schilfgaarde, R. Rat islet isograft function. Effect of graft volume and transplantation site. *Transplantation* 57:1010–1017; 1994.
  31. van Suylichem, P. T.; Wolters, G. H.; Van Schilfgaarde, R. The efficacy of density gradients for islet purification: A comparison of seven density gradients. *Transpl. Int.* 3:156–161; 1990.
  32. Wolters, G. H.; van Suylichem, P. T.; Van Deijnen, J. H.; Van Schilfgaarde, R. Factors influencing the isolation process of islets of Langerhans. *Horm. Metab. Res. Suppl.* 25:20–26; 1990.
  33. Wolters, G. H. J.; Fritschy, W. M.; Gerrits, D.; Van Schilfgaarde, R. A versatile alginate droplet generator applicable for microencapsulation of pancreatic islets. *J. Appl. Biomater.* 3:281–286; 1992.