Encapsulation significantly prolongs islet graft survival in the absence of immunosuppression. However, encapsulated islet graft survival is limited to periods of several months. Part of the encapsulated islet graft is affected by a non-progressive pericapsular overgrowth. To investigate whether macrophages on overgrown capsules affect neighboring non-overgrown encapsulated islets, encapsulated islets were studied during coculture. Encapsulated islet function, islet vitality and islet cell replication were assessed, as well as the mRNA expression of Bcl-2, Bax, iNOS, and MCP-1 in encapsulated islets after 48 hours of culture together with microcapsules with macrophage overgrowth. Overgrown capsules were retrieved from the rat peritoneum, three weeks after implantation of an encapsulated islet graft. Coculture was associated with inhibition of the stimulated insulin secretion, with decreased cell replication, and with increased cell necrosis, but not with apoptosis of encapsulated islet cells. mRNA expression levels in encapsulated islets after coculture were not different from controls, except for a decrease in Bax mRNA. We found a high level of nitrite, as an indicator of NO production, but not an increase in iNOS mRNA in islets. This, in combination with the absence of increase in MCP-1 mRNA and the lack of apoptosis, indicates that neither IL-1β nor TNF-α was responsible for the deleterious effects of coculture on encapsulated islets. Non-overgrown encapsulated islets are affected by the overgrowth on encapsulated islets in their close proximity. This overgrowth contains macrophages that produce nitric oxide which, rather than cytokines, may be held responsible for the deleterious effect on the neighbouring encapsulated islets.
Microencapsulation of pancreatic islets in alginate-poly-l-lysine-alginate capsules may avoid the necessity of permanent immunosuppressive drug therapy and opens perspectives for xenotransplantation in the treatment of insulin dependent diabetes mellitus. Microcapsules protect against cellular and antibody mediated rejection by separation of the graft from the host by a semi-permeable membrane. Although successes have been achieved in several animal models, the limited survival of encapsulated islet graft restricts the clinical applicability of the microencapsulation technique. Unfortunately, the occurrence of overgrowth cannot be avoided completely, although it can be restricted to approximately 10% of the microcapsules retrieved from the rat peritoneum (10). This apparent low percentage, however, conceals a considerable loss of 40% of the number of initially transplanted islets (11). From a simple quantitative point of view, pericapsular overgrowth should not impose a problem as long as the majority of the islet-containing microcapsules is not affected by overgrowth, and remains functional. However, macrophage-derived factors from the overgrown part of the graft may affect the non-overgrown part. Interleukin-1β (17.5 kD), Tumor Necrosis Factor-α (51 kD) and nitric oxide (NO) may pass the semi-permeable membrane and their cytotoxicity can lead to islet cell dysfunction and cell death (20). IL-1β can also increase Monocyte Chemoattractant Protein 1 (MCP-1) production in beta cells, which may amplify the attraction of macrophages and may thus contribute to the occurrence of overgrowth (3,4). Cytokines may also cause hyperproliferation of islets cells, which contributes to graft failure by limiting the life span of islet cells and by changing the beta/non-beta cell ratio (11). We hypothesize that the macrophages, which may exert deleterious effects on encapsulated islets, are located on overgrown capsules. Our present study examines the effect of macrophage overgrown capsules on encapsulated islets in an in vitro coculture system, which closely mimics the in vivo situation by using freshly encapsulated islets in the close proximity of overgrown capsules retrieved from the rat peritoneum.

**Materials and methods**

*Design of the study*

Encapsulated rat islets were cultured together with explanted microcapsules with macrophage overgrowth. After 48 hours, encapsulated islet function, islet vitality, and islet cell replication were assessed, as well as mRNA expression of Bcl-2, Bax, inducable NO Synthase (iNOS), and MCP-1. All analyses were
performed in the absence of overgrown capsules. The accumulation of IL-1β, TNF-α, and nitrite, as a measure for nitric oxide formation, in the culture medium was measured.

Encapsulated rat islets were prepared as described below. Microcapsules with overgrowth were harvested by peritoneal lavage, three weeks after implantation of an encapsulated islet graft in the peritoneum of a diabetic rat (allotransplantation). Microcapsules were explanted three weeks after implantation because overgrowth is established and complete within the first weeks after implantation (11,18). Islet containing and empty microcapsules with pericapsular overgrowth were put in culture together with encapsulated islets in a 1:9 ratio (coculture, *Figure 1*). The non-overgrown encapsulated islets were isolated a day before coculture and encapsulated on the first day of coculture.

**Animals**

Male inbred Albino Oxford rats served as islet donors for transplantation purposes (n=32, weighing 276 ± 24 gram) and coculture (n=36, weighing 298 ± 18 gram). Male Lewis rats served as recipients (n=6, weighing 287 ± 13 gram). All animals were purchased at Harlan (CPB, Zeist, The Netherlands) and received care in compliance with the guidelines of the local Animal Care and Use Committee following National Institutes of Health guidelines.

**Islet isolation**

Islets were isolated as described previously (28). Briefly, the donor pancreas was removed after infusion of 10 ml sterile Krebs-Ringer-Hepes (KRH) solution containing 10 % Bovine Serum Albumin (BSA) through the bile duct. The pancreas was chopped and digested using a two-stage incubation of 20 min at 37°C with successively 1.0 and 0.7 mg/ml collagenase (Boehringer P, Boehringer Mannheim, Germany). Islets were separated from exocrine tissue by centrifugation over a discontinuous Dextran gradient and further purified by handpicking. Islets were cultured overnight in non-treated petri-dishes (Greiner, Alphen a/d Rijn, The Netherlands) in portions of 100 islets per 25 cm² in CMRL 1066, 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% CO₂.

**Islet encapsulation**

Alginate (3.4 % w/v, 60 % mannuronic acid; Keltone LV, Kelco International, London, U.K.) was mixed with cultured islets to a concentration of 1,500 islets/ml. The microencapsulation procedure for transplantation and
for in vitro purposes was similar as described previously (9), with one modification. Encapsulated islets for coculture were incubated in 100 mM CaCl₂ for at least five but no more than seven minutes to avoid Ca²⁺ toxicity. CaCl₂ was then replaced with KRH and the islet-containing alginate beads (725-775 µm in diameter) were kept in KRH until all beads were generated. All beads were pooled and washed for one minute with KRH containing 2.5 mmol/l CaCl₂. PLL coating (poly-l-lysine-HCl, molecular weight 22,000; Sigma) was achieved by incubating for 10 minutes with 0.1 % PLL. Unbound PLL was washed away with Ca²⁺-free KRH (135 mmol/l NaCl) before applying the outer alginate layer by 10 minutes incubation in 10-times diluted alginate solution. Three successive washings with Ca²⁺-free KRH removed the excessive alginate.

**Diabetes induction and transplantation**

Two weeks prior to transplantation Lewis rats were injected with 75 mg/kg streptozotocin (Zonasar®, Pharmacia & Upjohn, MI, USA) through the tail vein. The animals became diabetic within 10 days and received an encapsulated islet graft after one week of hyperglycemia. Implantation was performed under isoflurane (Abbott Laboratories Ltd, U.K.) anesthesia (50% O₂ and 50% N₂O). Encapsulated islets were implanted intraperitoneally through a midline incision with an 18 mm gauge needle. After stitching, 10,000 I.E. Na-penicillin G (Yamanouchi Pharma, The Netherlands) was injected into the leg muscle.

![Figure 1](image.png)

**Figure 1.** Coculture; encapsulated islets cultured together with overgrown capsules in a 1:9 ratio (original magnification 10×4).
MACROPHAGE OVERGROWTH

Explantation and coculture

Three weeks after implantation, encapsulated islets were harvested by peritoneal lavage with warm RPMI (37 °C). Islet containing and empty microcapsules were regarded as overgrown if at least half of the capsule surface was covered with cellular overgrowth. The explanted graft was screened for overgrown capsules under a dissection microscope (SZ-6 Photo Light Microscope, Bausch & Lomb) and overgrown capsules were carefully selected by handpicking with a Pasteur pipette and gentle suction. Overgrown capsules were put in culture with freshly encapsulated islets in a ratio of 1:9, i.e. 10 capsules with overgrowth together with 90 freshly encapsulated islets. Control groups consisted of 90 freshly encapsulated islets only. Encapsulated islets were cultured in a 48-wells culture plate (tissue culture treated, Costar®, Cambridge, MA) for 48 hours, in 400 µl of CMRL 1066 with 5 % Normal Rat Serum, 8.3 mM glucose, 10 mM Hepes, 1 % Penicillin/Streptomycin at 37 °C in humidified air with 5 % CO₂.

Glucose challenge test

Encapsulated islets were tested for their glucose induced insulin response in three separate samples of 20 islets. Islets were pre-incubated for 45 minutes at 37 °C in 2 ml Krebs-Ringer-Bicarbonate (KRB) containing 0.25 % BSA and 2.75 mM glucose, gassed with 95 % O₂ and 5 % CO₂. The in vitro insulin secretion was then assessed by three consecutive incubations of (i) 45 minutes in 2.75 mM glucose in KRB, (ii) 45 minutes in 16.5 mM glucose and 0.1 % IBMX (Iso-Butyl-Methyl Xanthine, Sigma) in KRB, and (iii) 45 minutes in 2.75 mM glucose in KRB. At the end of each incubation, incubation media were completely removed for insulin determination by a radioimmunoassay for rat insulin (Linco, Ede, the Netherlands). The insulin secretory responses of 20 encapsulated islets during 45 minutes of incubation were expressed as nanograms of insulin/ml.

Vitality staining

Encapsulated islet vitality was assessed by discriminating between apoptotic, necrotic and vital cells. To this end, encapsulated islets were stained with 20 µg/ml Hoechst 33258 (bisbenzimide, Sigma) and 10 µg/ml Propidium Iodide (Sigma) in PBS for 10 minutes at 37°C, and scored as described by Saldeen (24). Islet cells with Hoechst (Ho) positive round nuclei were regarded as viable. Round nuclei were regarded necrotic if both Ho and propidium iodide (PI) positive. Highly condensed or fragmented nuclei, Ho positive or both Ho and PI positive, were identified as apoptotic cells. Counting was executed by an independent examiner who scored 700-800 cells from 8-12 islets per experiment at a 400x magnification (Leica DMLB/DC 300F). Scores should be regarded as estimations, since superposition of cells makes it difficult sometimes to determine the true
Cellular status.

**Immunohistochemical staining of BrdU**

Replication was assessed by estimating the percentage of islet cells that incorporated BrdU (Bromodeoxyuridine) during culture. BrdU labeling of the islets cells was performed by incubating the encapsulated islets with 10 µmol/l BrdU (Boehringer Mannheim, Germany) for 24 hours. BrdU-labeled islets were fixed in 2% paraformaldehyde, washed overnight with PBS containing 6% sucrose and dehydrated with aceton for 1 hour prior to GlycolMethAcrylaat (GMA)-embedding (all incubations at 4°C). Sections of 2 µm were treated with 0.1% trypsin (0.1 M Tris HCl, pH 7.8 + 0.1% CaCl₂) for 15 minutes at room temperature, stained with anti-BrdU (Goat anti BrdU with nuclease, Amersham, Uppssala, Sweden) and finally incubated with a secondary antibody conjugated with peroxidase (Rabbit anti Goat PO, DAKO, Glostrup, Denmark). Peroxidase activity was demonstrated by applying 3-amino-9-ethylcarbazole (AEC) containing hydrogen peroxidase. Hematoxylin was used for staining the cell nuclei. Per condition, approximately 1000 intact cells were counted. Necrotic and apoptotic cells were excluded from counting. Weakly stained cells were excluded from counting, since cell repair instead of cell replication could be responsible for staining the cell nuclei. The number of BrdU-positive cells was expressed as the percentage of the total number of BrdU-negative and BrdU-positive cells.

**RT-PCR**

Microcapsules were removed from the islets before analysis by shearing the encapsulated islets gently through a pipette, after a 30 minutes incubation step with 0.5 mM EDTA and 0.25% trypsin at 37°C (8). Batches of 60 decapsulated islets were washed three times with cold PBS and pellet frozen (-80°C). Total RNA was isolated using a Strataprep® Total RNA microprep kit (Stratagene, CA, USA) according to the manufacturers protocol. cDNA synthesis included an initial step in which RNA is incubated with T₅ VN oligo’s (0.5 µg) for 10 min at 70°C. Subsequently, first strand buffer, dNTP’s (final conc. 1 mM), DTT (final conc. 10mM), 200 U M-MLV Reverse Transcriptase (all from Gibco Brl) and 20 U recombinant RNasin® Ribonuclease Inhibitor (Promega) were added and incubated for 50 min at 37°C, after which the reaction was inactivated by a 15 min incubation at 70°C. Since only low amounts of total RNA can be isolated from 60 islets we standardized RT-PCR by using always one-third of the retrieved RNA, in the cDNA synthesis step. Two µl of cDNA was amplified by PCR in a buffer consisting of 0.2 mM dNTP’s, 1.5 mM MgCl₂, 1x PCR buffer and 1 U Taq DNA polymerase (all from Gibco Brl). Primer pairs (0.5 µM each) were added
after 3 min at 94°C (hot start). PCR was performed on a Mastercycler Gradient apparatus (Eppendorf, Germany) and cycles consisted of 94°C for 40 s, a primer-pair specific annealing temperature (see below) for 40 s and 72°C for 40 s. The number of cycles were selected to allow amplification within the linear range. The primer sequences, their annealing temperature, the number of cycles and their respective PCR fragment lengths were; β-actin (253 bp), 24 cycles, 55°C, 5’-AACACCCCAGCCATGTACG-3’, 5’-ATGTCACGC ACGATTTCCC-3’; Bel-2 (446 bp), 33 cycles, 58°C, 5’-GCTACGAGTG GGATACTGGAGA-3’, 5’-AGTCATCCACAGAGCGATGTT-3’ (25); Bax (300 bp), 30 cycles, 52 °C, 5’-AGGATGATTGCTGATGGATAC-3’, 5’-CACAAAGATGGGTCACTGT GTGC-3’ (25); iNOS (308 bp), 32 cycles, 57°C, 5’-GACTGCACAGAATGTT CCAG-3’, 5’-TGGCCAGATGGTTCCCTATT-3’ (5); MCP-1 (306 bp), 28 cycles, 57 °C, 5’-TTCACAGTTGCTGCCTGAGAC-3’, 5’-GTGCTGAAGTCCTTAGGGTTGA-3’. Each RNA sample was verified for absence of genomic DNA contamination by performing RT-PCR reactions in which the addition of the enzyme reverse transcriptase was omitted. Ethidium bromide-stained agarose gels were scanned on Image Master® VDS (Pharmacia Biotech, Upsala, Sweden) using LISCAP software. PCR abundance was quantified using Image Master 1D prime V2.00 (Amersham) and normalized for the abundance of the β-actin signal from the same cDNA.

**ELISA**

To verify the production of cytokines by macrophages, IL-1β and TNF-α levels were determined in culture medium samples. Protein levels were assessed by use of an ELISA kit (R&D systems, Minneapolis, USA) with an antibody directed against either IL-1β or TNF-α. A change in the color associated with the subversion of a substrate by a peroxidase-conjugate on a secondary antibody was assessed and used as a measure for cytokine presence.

**Nitrite measurement**

Nitrite formation was determined by use of the Griess reaction (15). An equal volume of the Griess reagent was mixed with 100 µl of medium and incubated for 10 minutes at room temperature. The absorbance at 540 nm was measured on an ultramicroplate reader (EL 808, Bio-Tek Instruments Inc.). Values were calculated from a standard curve ranging from 0.5 to 6.9 µg/ml.

**Statistical Analysis**

Results were statistically evaluated using a Mann-Whitney U test with a two-tailed distribution (* denotes a significance level < 0.05).
RESULTS

Islet vitality and function

The number of vital, necrotic and apoptotic cells was estimated as a measure for islet vitality and to determine cytotoxicity of macrophages (Figure 2). The percentage of vital cells in encapsulated islets after

![Figure 2. Encapsulated islets stained with the fluorescent dyes Ho and PI after normal culture (A) and after coculture (B). Small condensed cells, either Ho and/or PI positive, are apoptotic (arrow indicates apoptotic cell, original magnification 10x40).](image)

![Figure 3. Section of encapsulated islet containing one BrdU positive cell, which is indicated with an arrow (original magnification 10x40). Malformation of the microcapsule is the consequence of the dehydration step during the histological procedure.](image)
**MACROPHAGE OVERGROWTH**

**Islet vitality expressed as mean percentage (% ± sem) of vital cells, necrotic cells and apoptotic cells after 48 hours of culture (* = significant different compared to control).**

<table>
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<th>Control (n=5)</th>
<th>Coculture (n=5)</th>
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<tr>
<td>Vital</td>
<td>87 ± 3 %</td>
<td>79 ± 4 % *</td>
</tr>
<tr>
<td>Necrotic</td>
<td>11 ± 3 %</td>
<td>16 ± 4 % *</td>
</tr>
<tr>
<td>Apoptotic</td>
<td>2 ± 0 %</td>
<td>3 ± 1 %</td>
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Coculture was lower as compared to the percentage of vital cells in control islets. Coculture leads to an increase of the percentage of necrotic cells, but not to an increase of the percentage of apoptotic cells (Table 1).

Stimulated insulin secretion during a glucose challenge test and insulin produced during culture were determined as a measure of islet function. Stimulated insulin secretion of cocultured encapsulated islets was significantly lower when compared to stimulated insulin secretion of the control encapsulated islets (Figure 4). The amount of insulin in the culture medium was 8.1 ± 1.2 pg/ml for cocultured encapsulated islets, which was not different from controls (8.5 ± 1.1 pg/ml insulin).

**Islet cell replication**

BrdU incorporation in encapsulated islet cells during 24 hrs was assessed, in order to determine if cell proliferation is affected by the presence of overgrown capsules. The replication rate is the number of BrdU-positive cells expressed as the percentage of the total number of BrdU-negative and BrdU-positive cells (Figure 3). Since necrotic and apoptotic cells were excluded from counting, these values represent the percentage of replicating cells of the vital parts of the islets. Replication rate of encapsulated islets during coculture was 0.5 ± 0.2 % (mean ± sem), which was lower (P<0.05) as compared to controls (3.7 ± 2.0 %).

**mRNA expression levels**

To gain insight into molecular mechanisms that lead to loss of islet function and vitality, mRNA expression levels of several genes were analyzed. Bcl-2, Bax and iNOS are important intracellular mediators of cell death. The ratio of Bcl-2 and Bax expression is a critical determinant of cell fate, such that increased levels of Bcl-2 promote cell survival and increased levels of Bax promote apoptosis. Cytokine induced dysfunction and damage is strongly associated with
increased iNOS expression. Cytokines like IL-1β also enhance MCP-1 expression. The cytokine-induced elevation of iNOS and MCP-1 mRNA expression was verified in control experiments. We found that addition of 1000 U/ml IL-1β (R&D) and 10 U/ml TNF-α (R&D) to RIN-m5F beta cells lead to strong upregulation of both iNOS and MCP-1 mRNA (data not shown), which illustrates that the sensitivity of the RT-PCR method, as applied here, was adequate. mRNA levels of iNOS, MCP-1, Bcl-2, Bax, and Bcl-2/Bax ratio of the coculture experiments are shown in Table 2. mRNA levels of iNOS, MCP-1 and Bcl-2 in encapsulated islets during coculture were not different when compared to control groups. The level of Bax mRNA was significantly lower for cocultured encapsulated islets. This decrease was however not associated with a change in the Bcl-2/Bax ratio. Differences between coculture and control as observed with Bax only disappeared because Bcl-2/Bax ratios were calculated per individual experiment.

**IL-1β, TNF-α and nitrite**

Figure 5 shows the concentrations of IL-1β, TNF-α and nitrite, as found in the control and coculture medium. Concentration of cytokines in the coculture medium was higher as compared to controls, in which the concentration of cytokines approximated zero. Nitrite, as an indicator of nitric oxide production, was significantly higher in the medium of cocultured encapsulated islets as compared to the medium of control encapsulated islets.

![Figure 4. Islet function, expressed as insulin secretion capacity during a glucose challenge test. Basal, stimulation and recovery are insulin response to 2.75 mM glucose, 16.5 mM glucose + 0.1% IBMX and 2.75 mM glucose, respectively (* = P<0.05, n=5).](attachment:image.png)
TABLE 2. Relative mRNA expression levels (± sem) of iNOS, MCP-1, Bcl-2, Bax, and the Bcl-2/Bax ratio of control and cocultured encapsulated islets.

<table>
<thead>
<tr>
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<th>Control (n=5)</th>
<th>Coculture (n=5)</th>
</tr>
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<tbody>
<tr>
<td>MCP-1</td>
<td>2.82 ± 0.33</td>
<td>2.80 ± 0.64</td>
</tr>
<tr>
<td>iNOS</td>
<td>1.42 ± 0.21</td>
<td>1.63 ± 0.24</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>0.39 ± 0.15</td>
<td>0.33 ± 0.09</td>
</tr>
<tr>
<td>Bax</td>
<td>1.03 ± 0.08</td>
<td>0.73 ± 0.11*</td>
</tr>
<tr>
<td>Bcl-2/Bax</td>
<td>0.37 ± 0.13</td>
<td>0.39 ± 0.06</td>
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FIGURE 5. The concentration of IL-1β (A), TNF-α (B) and nitrite (C) in the culture medium (± sem, * = P<0.05, n=5).
Microencapsulation is an effective method to prevent cellular and antibody-mediated graft rejection. However, graft survival is limited to periods of several months, which may be caused by several factors of which two have been identified to be of main importance. One is related to limited diffusion of oxygen and nutrients to the encapsulated islet graft in the peritoneum. The other is related to overgrowth of microcapsules with macrophages and fibroblasts, which is caused by irregularities on the surface of alginate-poly-1-lysine microcapsules (10). The present study demonstrates that cellular overgrowth does not only suffocate islets within overgrown capsules, but also affects neighboring non-overgrown encapsulated islets through the production of toxic factors. The proximity of overgrown capsules leads to inhibition of the glucose stimulated insulin release, to a decrease of cell proliferation, and to an increase of necrosis in freshly encapsulated islets in vitro. Our results suggest that nitric oxide (NO), rather than cytokines (IL-1β and TNF-α), is responsible for deleterious effects of macrophage overgrowth on neighboring encapsulated islets. The suggestion that IL-1β is not responsible for the macrophage induced dysfunction is in line with the results of Kessler et al., who found that the functional activity in a coculture of encapsulated islets and macrophages is reduced. The addition of anti-IL-1β antibody to a coculture of macrophages and islets did not modify this loss of function (16). Wiegand et al. identified macrophage-derived NO as a cytotoxic factor in the destruction of encapsulated islets (27). They showed that macrophages that were previously activated in vivo, but not resident macrophages, exert this cytotoxicity during an in vitro coculture. In addition, the present study shows that macrophages on overgrown capsules retrieved from the rat peritoneum are responsible for loss of function and vitality during coculture in vitro, and supports the observation that NO is the cytotoxic factor. Absence of toxicity by cytokines can be attributed to protective effects of the semi-permeable membrane, which prevents, or at least hinders the passage of IL-1β and/or TNF-α (2,7,29).

The suggestion that NO rather than cytokines is involved in the deleterious effects of overgrown capsules on encapsulated islets is based on a number of observations. IL-1β inhibits stimulated insulin secretion and induces islet cell death, an effect that is potentiated by TNF-α. In rat islet cells, the cytokine-induced dysfunction and damage are mediated by inducible NO synthase (iNOS)(6,13). We found increased levels of IL-1β and TNF-α in the coculture medium, but not an increase of iNOS mRNA in islet cells, which indicates that the damage and dysfunction of the encapsulated islets was not mediated by cytokine-induced iNOS. Not only the lack of iNOS mRNA, but also the absence
of elevated MCP-1 mRNA levels indicate that encapsulated islets remained unaffected by cytokines, since II-1β has been shown to be a strong inducer of MCP-1 expression in rat islet cells (3,4). MCP-1 expression is not associated with iNOS expression and not mediated by NO (4). Thus, our results with iNOS and MCP-1 expression levels - independent of each other – indicate that the deleterious effects of macrophages were not mediated by cytokines.

Since we found increased levels of nitrite in the culture medium and no increased iNOS expression in encapsulated islet cells, it is likely that NO was produced by macrophages on overgrown capsules. Macrophage-derived NO may well explain all observed changes of the encapsulated islets. The replication rate of encapsulated islets during coculture was 0.5%, which was significantly lower compared to the 3.7% of the control encapsulated islets. This decrease can be attributed to NO, which is known to suppress cell proliferation (12,17,22). Vitality staining results showed an increase in necrosis and not in apoptosis, which is also in line with the notion that NO rather than cytokines affect encapsulated islets. The predominant mode of cell death induced by cytokines, either as direct mediators or by inducing iNOS, is apoptosis. NO has a more relevant role for the necrotic than for the apoptotic component of beta cell death (14,21). High NO concentrations will induce necrosis in cells lacking sufficient energy and reduction equivalents, a cell death pathway typically seen in pancreatic islet cells and neurons (1,19). Indeed, in the present study, high levels of nitrite were associated with necrosis. Furthermore, we did not find an increase but a decrease in Bax, which is in line with the observed necrosis. The decreased Bax expression, in combination with constitutive levels of Bcl-2 mRNA, may even lead to an inhibition of apoptosis (26).

Our results have implications for the interpretation of the process that leads to graft failure. Apparently, the deleterious effects of macrophage overgrowth extend beyond the overgrown capsules to neighboring non-overgrown encapsulated islets. This emphasizes the need to prevent pericapsular overgrowth. However, it is unlikely that overgrowth can be avoided completely. Therefore, additional strategies to protect encapsulated islets against the effects of macrophages are required, which in view of our results should aim at protecting the islets against NO toxicity. Several approaches have been studied by others (1,23). The use of microcapsules provides the opportunity to co-encapsulate islets with other cell types that may protect against the toxic effects of NO. Coencapsulation with erythrocytes has been shown to effectively protect the islets within the capsules from NO toxicity (27).

In conclusion, a small portion of overgrown capsules in the close proximity of non-overgrown capsules has deleterious effects on encapsulated islet function, vitality and proliferation. Macrophage derived NO, rather than cytokines, may
be held responsible for the inhibition of stimulated insulin secretion, inhibition of cell replication, and an increase of necrosis, but not of apoptosis. This emphasizes the need to develop capsules that are completely devoid of pericapsular overgrowth. Alternatively, protection of encapsulated islets against exogenously produced NO may contribute to an improved encapsulated islet graft survival.
REFERENCES


