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## Factors Influencing Insulin Secretion From Encapsulated Islets

Bart J. de Haan, Marijke M. Faas, and Paul de Vos

Immunoendocrinology, Department of Pathology and Laboratory Medicine, Section of Medical Biology, University of Groningen, Hanzeplein 1, 9700 RB Groningen, The Netherlands

Adequate regulation of glucose levels by a microencapsulated pancreatic islet graft requires a minute-to-minute regulation of blood glucose. To design such a transplant, it is mandatory to have sufficient insight in factors influencing the kinetics of insulin secretion by encapsulated islets. The present study investigates factors influencing the glucose-induced insulin response of encapsulated islets *in vitro*. We applied static incubations and did the following observations. (i) Small islets (90–120  $\mu\text{m}$ ) showed a similar instead of a lower glucose-induced insulin response, suggesting that inclusion of only small islets, which are associated with lower protrusion and failing rates, has no consequences for the functional performance of the graft. (ii) A capsule diameter of 800  $\mu\text{m}$  showed identical rather than lower glucose-induced insulin responses as smaller, 500- $\mu\text{m}$  capsules. (iii) Capsule membranes constructed with a conventional permeability interfered with diffusion of insulin, as illustrated by a lower response of islets in capsules with a 10-min poly-L-lysine (PLL) membrane than islets in capsules with a 5-min PLL membrane. (iv) Irrespective of the tested porosity, the capsules provided sufficient immunoprotection because the 10-min PLL membranes did block diffusion of the cytokines IL-1 $\beta$  (17 kDa) and TNF- $\alpha$  (70 kDa) while the 5-min PLL membranes interfered with the diffusion of the vast majority of the cytokines. We conclude that capsules containing small islets (90–120  $\mu\text{m}$ ) and a membrane with a lower permeability than routinely applied is preferred in order to obtain a graft with adequate glucose-induced insulin responses.

Key words: Alginate; Islets; Transplantation; Poly-L-lysine; Encapsulation

### INTRODUCTION

Immunoprotection involves envelopment of tissue in a semipermeable membrane to protect donor cells against antibodies and cytotoxic cells of the host immune system. This immunoprotection by encapsulation allows for successful transplantation of cells in the absence of immunosuppression. A commonly used procedure for immunoprotection is microencapsulation of tissues in alginate-poly-L-lysine (PLL)-based capsules as originally described by Lim and Sun (19). During recent years, important advances have been made with this technology. The first allotransplantations in humans with encapsulated parathyroid cells and pancreatic islets have been successfully performed (15,24). Also, successful xenotransplantation of islet grafts has been shown in both chemically induced and autoimmune diabetic rodents (13,21,34), dogs (23,32), and monkeys (25,38).

A major requirement for clinical application of encapsulated pancreatic islets is that the graft adequately regulates glucose levels. Although this is a factor that may contribute to the variable success rates of encapsu-

lated grafts (1,8,13,21,23), it has been the focus of not more than a few studies (1,6,8,11,26). The diffusion kinetics for encapsulated islets has been shown to be different from islets in the native pancreas. A delay in insulin response after a glucose stimulus has been observed both *in vitro* (33) and *in vivo* (1,6,11). Plausibly, this delayed insulin response is caused by interference of the capsule membrane with optimal exchange of glucose and insulin (26). The rate of diffusion over the capsule membrane is determined by surface/volume ratio of the capsules. This implies that when the capsule diameter is small the ratio and thus the diffusion rate will be higher. Therefore, small capsule diameters are preferred for transplantation of encapsulated islets. Yet, a major obstacle in application of small-diameter capsules is the high amount of protruding islets, which cause rejection and subsequent failure of the graft (5,7). A conceivable approach to reduce protrusion is the use of only small islets for encapsulation. However, it is not known whether this has a drawback on the function of the graft because small islets are assumed to produce less insulin than large islets.

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Address correspondence to B. J. De Haan, Ph.D., Pathology and Laboratory Medicine, Section of Medical Biology, University of Groningen, Hanzeplein 1, 9700 RB Groningen, The Netherlands. Tel: (31) (50) 3619826; Fax: (31) (50) 3619911; E-mail: B.J.DE.HAAN@MED.RUG.NL

In the present study we compare the glucose-induced insulin response of encapsulated islets containing islets with different sizes. In addition, we identified some other factors influencing the glucose-induced insulin response of encapsulated pancreatic islets. Identification of such factors appears mandatory for modifying the alginate-PLL microencapsulation procedure in order to allow adequate responses of the microencapsulated tissue.

## MATERIALS AND METHODS

### *Animals*

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

### *Islet Isolation*

Islets were isolated as previously described (36). Briefly, under ether anesthesia, the abdomen was opened and the common bile duct was cannulated under non-sterile conditions. The donor pancreas was distended with 10 ml sterile Krebs-Ringer-HEPES supplemented with 25 mmol/L HEPES buffer (KRH) 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 and 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, USA). Islets were separated from exocrine tissue by centrifugation over a discontinuous dextran gradient (30) and further purified by handpicking.

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  $\mu\text{m}$ . For determination of the diameters we measured at least 150 islets.

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  $\text{cm}^2$  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% penicillium/streptomycin] at 37°C in humidified air containing 5%  $\text{CO}_2$ .

### *Encapsulation*

After culture, islets were washed three to five times with RPMI containing 10% FCS and were subsequently suspended in sterile filtered (0.2  $\mu\text{m}$  filtration) 3–3.3% purified alginate solution. The alginate solution was con-

verted into droplets using an air-driven droplet generator as previously described (37). Subsequently, the alginate droplets were transformed to alginate beads by gelling in a 100 mM  $\text{CaCl}_2$  (10 mM HEPES, 2 mM KCl) solution for at least 5 min (14). After gelation the beads had a diameter of 450–550  $\mu\text{m}$  when 800- $\mu\text{m}$  capsules were produced and 350–400  $\mu\text{m}$  when 500- $\mu\text{m}$  capsules were produced. The Ca-alginate beads were suspended for 3 min in KRH containing 2.5 mmol/L  $\text{CaCl}_2$ . A PLL membrane was formed by suspending the alginate beads in 0.1% PLL solution for 5 or 10 min (poly-L-lysine-HCl, MW: 22,000, Sigma). Nonbound PLL was removed by three successive washings during 3 min with  $\text{Ca}^{2+}$ -free KRH containing 135 mM NaCl. The outer alginate layer was subsequently applied by 5-min incubation in 10 times diluted alginate solution.

### *Insulin Secretion During Glucose Challenge*

In static incubation experiments, islets were tested in four separate samples of 10 islets each. To minimize the variability of the mean insulin responses, we meticulously selected islets with diameters between 90 and 120  $\mu\text{m}$ . In some experiments we used other islet diameters, as indicated in the Results section. The encapsulated islets were preincubated for 45 min in 2 ml Krebs-Ringer-bicarbonate (KRB), gassed with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ , containing 0.25% BSA and 2.75 mM glucose. The quantitative 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. These incubations were performed in a Labline metabolic incubator at a stirring rate of 120 cycles/min at 37°C. At the end of each incubation, the incubation media were removed and frozen for insulin determination. The insulin secretory responses were expressed as nanogram of insulin  $\text{mL}^{-1}$  10 islets $^{-1}$  45 min $^{-1}$ .

The incubations with varying experimental conditions were simultaneously performed with one batch of islets on 1 day. These incubations were always performed threefold to reduce variations in the individual experiments. The experiments were repeated five to eight times.

### *Cell Culture*

The nr8383 macrophage cell line was cultured in 60-ml culture flasks containing RPMI-1640 with 10% FCS, 8.3 mmol/L glucose, 10 mmol/L HEPES, and 1% penicillium/streptomycin. The cultures were kept at 37°C in humidified air containing 5%  $\text{CO}_2$ .

The nr8383 macrophage cell line was encapsulated in a novel two-step procedure. First, the cells were immobilized in a small alginate bead of 200  $\mu\text{m}$ . Therefore, LPS-stimulated (0.5  $\mu\text{g}/\text{ml}$  for 1 h) nr8383 cells were mixed in a ratio of 30 million cells per 1 ml alginate.

The alginate/nr8383 suspension was converted into droplets using an air-driven droplet generator (37). Droplets of approximately 200  $\mu\text{m}$  were produced and allowed to gel for 5 min in 100 mM  $\text{CaCl}_2$  (10 mM HEPES, 2 mM KCl). High numbers of cells were protruding from these small beads. In the second step, the cells containing Ca-alginate beads were resuspended in alginate for encapsulation without protrusion of cells. To this end, the 200- $\mu\text{m}$  Ca-alginate beads were suspended in a 2 ml 3.4% alginate solution for a second envelopment in beads. The alginate containing immobilized nr8383 cells was converted into droplets and allowed to gel for 5 min in 100 mM  $\text{CaCl}_2$  (10 mM HEPES, 2 mM KCl). After gelation the beads had a diameter of 550–600  $\mu\text{m}$ . The beads were subsequently subjected to the encapsulation procedure as described in the encapsulation section.

To test the permeability of the capsules for the nr8383-derived IL-1 $\beta$  and TNF- $\alpha$ , the beads were coated with three different PLL time periods to obtain three different permeabilities. Therefore, the beads were divided into three groups and incubated for 0, 5, and 10 min with 0.1% PLL.

The experiments were always performed in triplicate to reduce variations in the individual experiments. To this end, three separate samples of 100 capsules containing macrophages were incubated in 24-well plates and cultured for 48 h in RPMI-1640 with 10% FCS, 8.3 mmol/L glucose, 10 mmol/L HEPES, and 1% penicillin/streptomycin at 37°C in humidified air containing 5%  $\text{CO}_2$ . At the end of the experiments the incubation media were removed for assessment of IL-1 $\beta$  and TNF- $\alpha$  production. Data are expressed as ng/ml.

#### Determinations

Insulin was determined by a radioimmunoassay for rat insulin (Linco, St. Charles, MO, USA) using rat insulin as a standard. IL-1 $\beta$  and TNF- $\alpha$  were determined by ELISA (R&D, Abingdon, UK) using rat IL-1 $\beta$  and rat TNF- $\alpha$  as a standard.

#### 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

The diameters of isolated islets varied between 60 and 300  $\mu\text{m}$  (Fig. 1). The effect of the islet diameter on the insulin secretion before and after encapsulation was tested with diameter populations of 90–120, 150–180, and of 210–300  $\mu\text{m}$  because these islet diameters were present in sufficient numbers for static incubation testing. As shown in Figure 2, the three diameter popula-

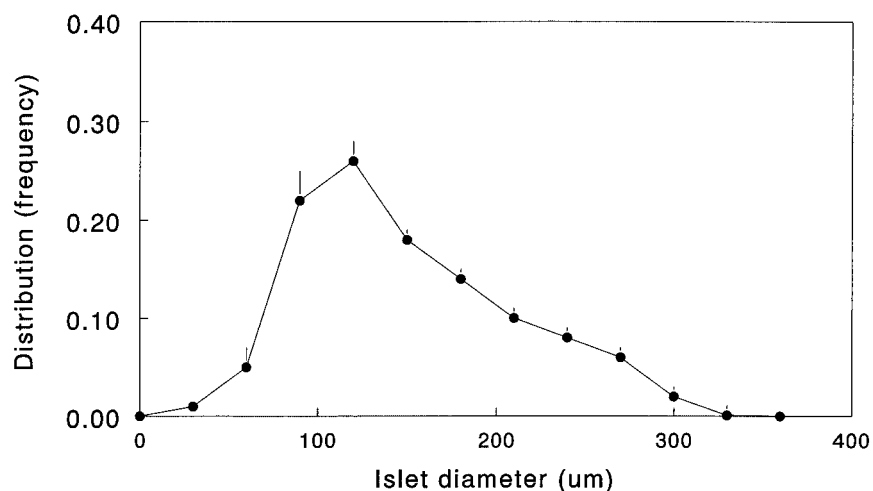
tions tested showed identical instead of a size-dependent difference in their glucose-induced insulin response. The 210–300- $\mu\text{m}$  islets produced slightly more insulin after stimulation with glucose, but this never reached statistical significant difference.

Surprisingly, the presence of a capsule around the islets reduced both the basal and the stimulated glucose-reduced insulin response. Theoretically, this reduction in insulin response can be caused by two factors. First, the encapsulation procedure may have interfered with the viability of the islet tissue because reportedly (14) some steps in the procedure interfere with adequate function of the islets. Secondly, and more plausibly, the presence of the capsule may have interfered with the exchange of glucose and insulin over the capsule membrane (3,8,33). To investigate whether the observed reduction in insulin response was caused by a harmful step in the encapsulation procedure or by the presence of a capsule around the islet we removed the capsule. To this end, the capsule was manually disrupted and removed before the insulin-secreting capacity of the so-called deencapsulated islets were tested. As shown in Figure 3, the insulin response of deencapsulated islets was restored and statistically significantly higher ( $p < 0.02$ ) than that of islets still enveloped within the capsules.

Next, we investigated whether the interference of the capsule membrane with adequate glucose-induced insulin responses was caused by the relative large capsule size of 800  $\mu\text{m}$  because the capsule size has been reported to be an important factor influencing the glucose-induced insulin response of encapsulated islets (Fig. 4). Therefore, we tested and compared islets encapsulated in large (800- $\mu\text{m}$ ) capsules and islets encapsulated in small (500- $\mu\text{m}$ ) capsules. In these tests the capsules were incubated with 5 min of PLL instead of the 10 min applied in the experiments in Figure 2.

As shown in Figure 5, the basal and stimulated insulin secretion of islets in 500- and 800- $\mu\text{m}$  capsules showed similar if not identical insulin levels. Evidently, the reduction in capsule diameter from 800 to 500  $\mu\text{m}$  did not bring about the expected increase in secretory capacity of encapsulated islets.

A surprising observation in the previous experiment was done when we compared the quantitative insulin secretion of the encapsulated islets in the set of experiments in Figure 5 with those of the first set (Fig. 2). In the experiments presented in Figure 5 we found a basal and stimulated insulin secretion of encapsulated islets that was not lower but identical to that of free islets (106  $\pm$  9 vs. 98  $\pm$  11 for basal, and 308  $\pm$  29 vs. 296  $\pm$  42 for stimulated). Because in the previous experiments we had set the incubation time with PLL to 5 min instead of the routinely applied 10 min, we questioned whether the PLL incubation time was an unidentified factor deter-



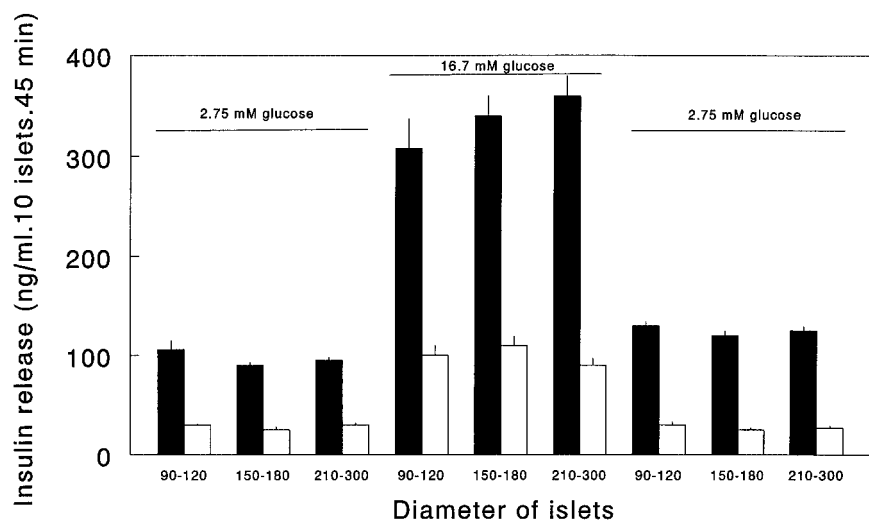
**Figure 1.** Distribution of the islet diameters in a collagenase digested pancreas ( $n = 12$ ). Values represent mean  $\pm$  SEM.

mining the capacity of encapsulated islets to respond adequately to a glucose load.

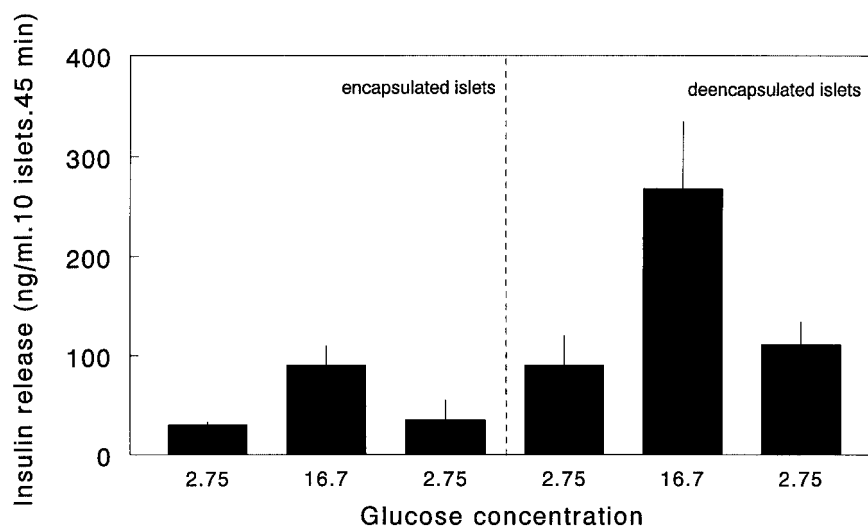
Therefore, we tested and compared in a next set of experiments (i) free islets, (ii) islets in capsules treated for 5 min with PLL, and (ii) islets in capsules treated for 10 min with PLL. As shown in Figure 6, free islets and islets in capsules treated for 5 min with PLL elicited similar amounts of insulin during incubation in both low and high glucose while islets in capsules treated for 10 min with PLL showed a profound reduced response.

Finally, we questioned whether the improved results

with 5-min PLL incubation could be explained by a lowered permeability of the capsules. Therefore, the effect of the PLL reaction time on the diffusion of low and high molecular molecules was investigated with the cytokines IL-1 $\beta$  (17 kDa) and TNF- $\alpha$  (70 kDa). These permeability testings were done by assessing the diffusion of these cytokines from encapsulated nr8383 macrophages. As shown in Figure 7, we found that decreasing the conventional permeability of the membrane by reducing the PLL incubation time 5 min, the porosity of the membrane for IL-1 $\beta$  increased from <4% to 26%,



**Figure 2.** Glucose-induced insulin response of free ( $n = 6$ , filled bars) and microencapsulated ( $n = 6$ , open bars) rat islets with varying diameters. Alginate-PLL capsules were 800  $\mu\text{m}$ . Values represent mean  $\pm$  SEM.



**Figure 3.** Glucose-induced insulin response of microencapsulated ( $n = 5$ , left) rat islets and islets from which the microcapsule (i.e., deencapsulated) was removed ( $n = 6$ , right). Alginate-PLL capsules were 800  $\mu\text{m}$ . Values represent mean  $\pm$  SEM.

and for TNF- $\alpha$  from <2% to 28%, illustrating a profound effect of this seemingly minor modification in PLL reaction time on the permeability of the capsules.

### DISCUSSION

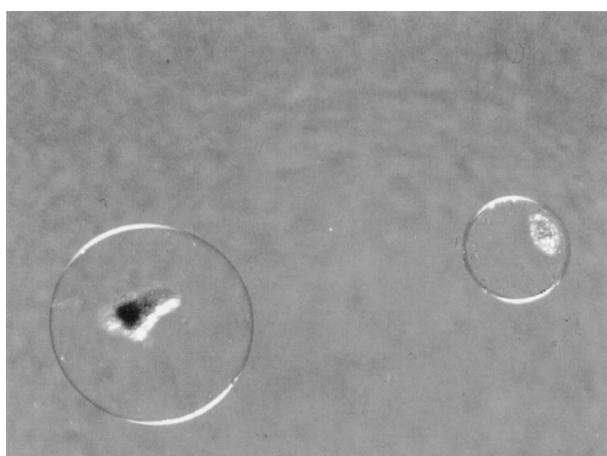
To provide a minute-to-minute regulation of blood glucose levels by transplanted microencapsulated islets, the kinetics of insulin secretion by encapsulated islets are of crucial importance. This study clearly demonstrates that the responses of encapsulated islets are influenced by the permeability of the capsule membrane and not by

the islet diameters or capsule diameter in the routinely applied 500–800- $\mu\text{m}$  range.

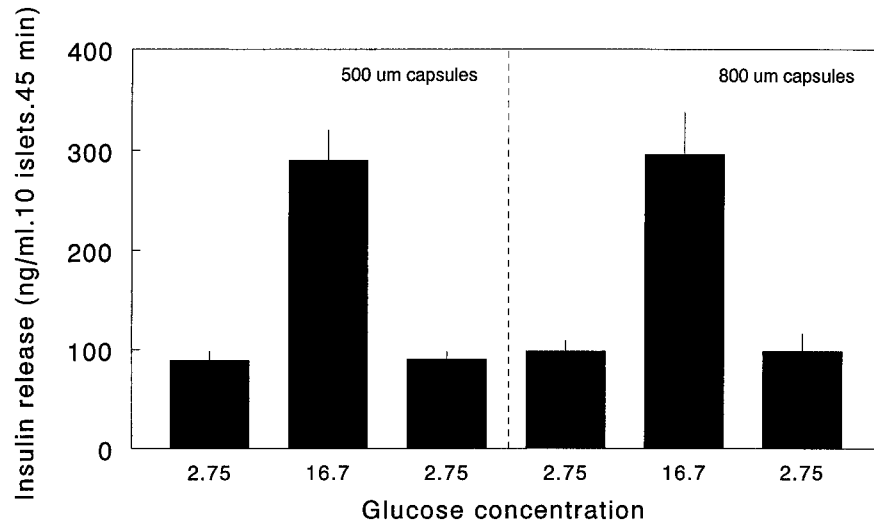
The finding that the glucose-induced insulin response of encapsulated islet is not influenced by the capsule's diameter does not corroborate the findings of Chicheportiche et al. (3). These authors observed an almost complete abrogation of the glucose-induced insulin response when the capsules size was increased from 300 to 800  $\mu\text{m}$ . A possible explanation for this discrepancy in observations is the location of the islets in the capsules, which was more peripheral in the capsules applied in the present study (12). This implies a shorter diffusion distance for both glucose and insulin over the capsule membrane and, therefore, may facilitate the responses of the islets in the large, 800- $\mu\text{m}$  capsules.

During our comparison of the glucose-induced insulin responses of islets with different sizes, we surprisingly found similar instead of size-dependent differences in insulin secretion. This lack of effect of islet size on insulin secretion could probably be explained by the fact that in isolated islets the glucose is penetrating the islets by passive diffusion instead of by active transport to each individual islet cell by the bloodstream. This passive diffusion of glucose is predictably less efficient than active transport to the islet cells by the bloodstream as in normal, vascularized islets in the native pancreas (2,20). Consequently, the glucose around large isolated islets will only stimulate islet cells in the periphery rather than the more centrally located islet cells.

Our observations that islets of different sizes show similar responses confirm the findings of Colella et al.



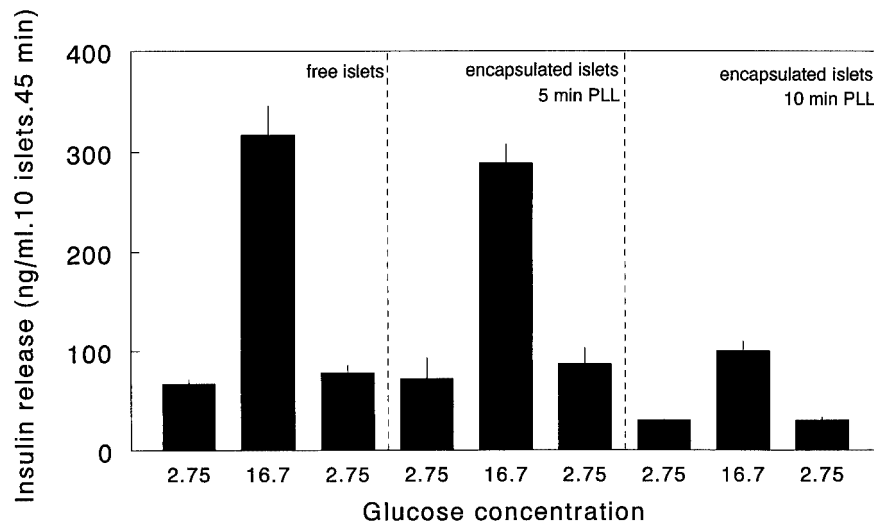
**Figure 4.** Islets in capsules of 500 and 800  $\mu\text{m}$ . Note the large difference in volume of the capsules (original magnification  $\times 10$ ).



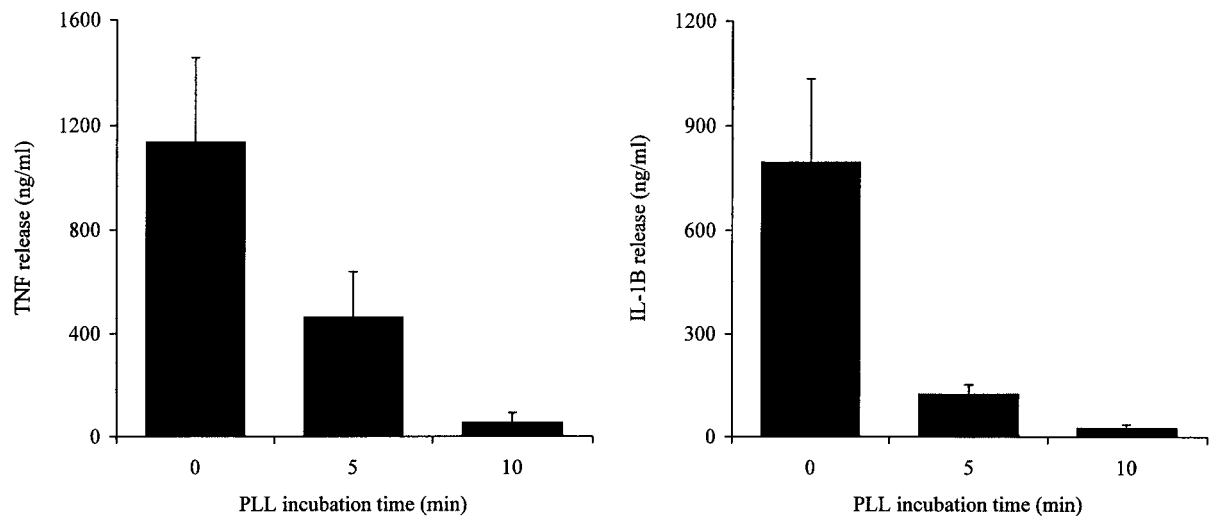
**Figure 5.** Effect of capsule diameter on glucose-induced insulin response of microencapsulated rat islets in capsules of 500 μm (*n* = 8, left) and 800 μm (*n* = 8, right) Values represent mean ± SEM.

(4), who observed a similar insulin secretory response in spite of a twofold difference in DNA content. However, our observations do not corroborate the findings of Jahr et al. (16) and Reaven et al. (22). This could be explained by a difference in the experimental set up. Jahr et al. studied the insulin secretion of islets only at a continuous high glucose level instead of investigating the responses during glucose challenge at low and a subsequent high glucose level (16). This is essential because the former does not necessarily represent the physiologi-

cal response but an adaptation of the basal insulin secretion to a continuous change in environment instead of an increased response to a sudden change in metabolic demand. Also, the time period in which the challenge is performed may contribute to the difference in findings. The magnitude of the response in static incubations is dependent on diffusion of glucose into the islets. This implies that this process is complete in a shorter period of time in small than in large islets and may explain why in static incubation systems with short incubation



**Figure 6.** Effect of permeability of microcapsules on glucose-induced insulin response of microencapsulated rat islets. Free islets (*n* = 6, left), islets in capsules with low permeability induced by 5-min incubation in PLL (*n* = 6, middle), and islets in capsules with routine, commonly applied permeability by 10-min incubation in PLL (*n* = 6, right). Values represent mean ± SEM.



**Figure 7.** TNF- $\alpha$  and IL-1 $\beta$  release of encapsulated LPS-stimulated nr8383 macrophages ( $n = 4$ ) with different permeabilities of the capsules by varying the PLL incubation times. Values represent mean  $\pm$  SEM.

periods small islets respond with a similar insulin secretion, because not all cells in the larger islets have participated. Consequently, when the incubations are performed for a longer period of time a higher response is to be expected. This argumentation is supported by the observations of Reaven et al. (22), showing a somewhat higher response of the larger islets when the authors applied a 33% longer incubation period of 60 min instead of the conventional 45 min. In our experimental design we applied a 45-min incubation period because in vivo, in successful graft recipients, glucose levels usually return to basal in this time period (9).

The observation that encapsulated small, 90–120- $\mu\text{m}$  islets show similar instead of lower glucose-induced insulin responses than 150–180- and 210–300- $\mu\text{m}$  islets suggests that the inclusion of only small islets in encapsulated grafts will have no consequences for the functional performance of the grafts. This is a pertinent consideration because small islets are associated with less protrusion (5,7,27) and, therefore, a lower rate of failing encapsulated islets. Another advantage of application of small islets is the reduction of problems associated with insufficient nutrition and central necrosis of the encapsulated islets with sizes larger than 150  $\mu\text{m}$  (10). This necrosis has been shown to be a major cause of failure of encapsulated grafts (8,10). However, the effects of application of only the small rather than the large islets on longevity of encapsulated islet grafts requires some further research considerations because longevity of the graft is determined by the size of the graft volume (28,29), which is mainly determined by the number of large islets in the transplant (29,35,36).

The dramatic effect of lowering the permeability on

the glucose-induced insulin response by decreasing the PLL incubation time only 5 min is rather surprising, because our and other previous studies have shown that capsules treated with similar incubation times with similar types of PLL molecules are permeable for substances up to 120 kDa (5,17,31). Notably, previous studies on permeability of capsules were performed with linear, nonionic molecules (mostly dextran), which have diffusion characteristics far different from that of nonlinear and charged proteins such as insulin. Our present data suggest that it is mandatory to study permeability of capsules by assessing the diffusion of relevant bioactive factors rather than by measuring exchange of irrelevant model molecules such as dextran.

The above also holds for diffusion of molecules that are involved in rejection of islets such as chemokines and cytokines. Based on the molecular weight of the cytokines, it has been assumed that conventional immunisolating capsules cannot prevent the entry of the majority of chemokines and cytokines (18). In the present study, we showed for the first time that in spite of their low molecular weight the cytokines IL-1 $\beta$  (17 kDa) and TNF- $\alpha$  (70 kDa) cannot readily diffuse over the capsule membrane. Capsules membranes treated for 10 min with PLL showed virtually no permeability for IL-1 $\beta$  and TNF- $\alpha$  while capsule membranes treated for 5 min allowed passage of only a portion of less than 30% of the total amount of cytokines. After deencapsulating the macrophages from the 5- and 10-min PLL-treated capsules, the cytokine levels reached the values of the non-treated PLL capsules (data not shown). This illustrates that the PLL treatment does not interfere with the cytokine production by the macrophages but that the mem-



brane interferes with the diffusion of the cytokines. Capsule membranes treated for 5 min with PLL is probably sufficient to protect the islet cells against cytotoxicity because the deleterious effect of the cytokines depends on their final concentration and combined presence. Also, successful transplantation has been shown with these low-permeability capsules (19).

Our findings emphasize that an optimal capsule configuration is required for adequate functional performance of the encapsulated islets and, consequently, a maximum chance for successful performance of the encapsulated islet transplant.

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