We describe a new technique for microencapsulation with high–mannuronic acid (high-M) alginate crosslinked with BaCl$_2$ without a traditional permselective component, which allows the production of biocompatible capsules that allow prolonged survival of syngeneic and allogeneic transplanted islets in diabetic BALB/c and NOD mice for >350 days. The normalization of the glycemia in the transplanted mice was associated with normal glucose profiles in response to intravenous glucose tolerance tests. After explantation of the capsules, all mice became hyperglycemic, demonstrating the efficacy of the encapsulated islets. The retrieved capsules were free of cellular overgrowth and islets responded to glucose stimulation with a 5- to 10-fold increase of insulin secretion. Transfer of splenocytes isolated from transplanted NOD mice to NOD/SCID mice adoptively transferred diabetes, indicating that NOD recipients maintained islet-specific autoimmunity. In conclusion, we have developed a simple technique for microencapsulation that prolongs islet survival without immunosuppression, providing complete protection against allorejection and the recurrence of autoimmune diabetes. *Diabetes* 50:1698–1705, 2001

Rapid Publication

**Complete Protection of Islets Against Allorejection and Autoimmunity by a Simple Barium-Alginate Membrane**


Islet transplantation represents an important alternative for the treatment of type 1 diabetes but still requires immunosuppressive agents with their serious side effects (1). One approach to avoid such treatment is to protect islets from the host’s immune system with a semipermeable, biocompatible membrane (2,3). Transplantation of islets contained in alginate-poly-L-lysine (PLL) capsules was first described by Lim and Sun (4). Numerous studies have shown successful reversal of diabetes by transplantation of islets enclosed in alginate-PLL capsules in streptozotocin-induced animals (5–9). However, limited success has been reported in spontaneously diabetic NOD mice, a model of autoimmune diabetes (10–13). Various factors have been implicated in the failure of encapsulated islets. A cellular reaction surrounding the capsules has often been observed, which could lead to depletion of oxygen and nutrients (14) or production of toxic cytokines (15). This accumulation of cells could be due to an immune response to the contained islets or to bioincompatibility of the capsular materials (16,17). Failure might also be attributed to problems with β-cell viability in the capsules (18).

Protection of porcine islets remains a goal of encapsulation, but xenografts might be more difficult to protect than allografts, as suggested by studies performed with permeable polymer membranes (19,20). This concept is important because of the improved prospects for obtaining an abundant supply of human β-cells from precursor cells (21,22). The goal of this study was to determine whether stable biocompatible alginate microcapsules without a permselective component, such as PLL or polyethylene-glycol, would be able to protect mouse islets against allorejection and autoimmunity.

**RESEARCH DESIGN AND METHODS**

**Islet isolation.** Islets were isolated from male NOD mice (4 to 6 weeks old; Taconic Farms) and B6AF1 mice (6 to 8 weeks old; Jackson Laboratories, Bar Harbor, ME). Pancreases were infused via the common bile duct with rodent Liberase RI (Roche, Indianapolis, IN) and digested for 30 min at 37°C. Islets were purified on a discontinuous ficoll gradient (Sigma, St. Louis, MO), handpicked under a stereomicroscope, and then cultured overnight in RPMI-1640 (Life Technologies, Rockville, MD) with 11 mmol/l glucose supplemented with 10% fetal calf serum (Mediatech, Herndon, VA), penicillin-streptomycin (100 units/ml and 100 µg/ml, respectively; Life Technologies), and glatamine (2 mmol/l; Life Technologies) at 37°C in humidified air atmosphere containing 5% CO$_2$.

**Microencapsulation.** Encapsulation was carried out with slight modification of a previously described technique (23) in conditions that minimize the presence of endotoxin. Briefly, alginate, alone or mixed with islets, was loaded in a tube connected to a pump and an air-driven droplet generator. Droplets fell into a cationic solution that crosslinked the alginate to form gel microcapsules.

**Empty capsules.** Alginates differ in their content of mannuronic and guluronic acids. To select the most biocompatible alginate, two different highly purified alginates with minimal endotoxin contamination were used. The first alginate, used at a concentration of 1.6% (wt/vol), was characterized by a high guluronic content (70%, high G alginate; provided by Dr. D. Scharp, Novocell, Irvine, CA). The second alginate, used at a concentration of 3.3% (wt/vol) was characterized by a high mannuronic acid content (61%, high M alginate; donated by John Holahan, Pharmacia, Peapack, NJ). CaCl$_2$ (100 mmol/l) and BaCl$_2$ (10 mmol/l) were used as crosslinkers. When CaCl$_2$ was used, half of the capsules were coated with PLL 20 kDa (0.1%; Sigma) followed by a third layer of 10× diluted alginate.

**Islet microencapsulation.** After overnight culture, islets were suspended in high–mannuronic acid (high-M) alginate at a concentration of 2.000 islets/ml. Alginate microcapsules (900–1,100 µm in diameter) were formed by crosslinking with BaCl$_2$ (10 mmol/l). Microencapsulated islets were cultured overnight in Ultraculture medium (glucose concentration: 20 mmol/l; Biowittaker,
Mice were considered normoglycemic (200 mg/dl) or hyperglycemic (300 mg/dl) and then once a week for the remainder of the study. Glucose levels were measured by tail vein puncture and glucose tolerance tests (IVGTTs) (1.5 g/kg of body wt) were performed at 49, 105, and 210 days after transplantation. Blood samples were obtained from the tail vein to measure glucose levels and C-peptide levels by radioimmunoassay (RIA). Plasma samples were immediately frozen and stored at −80°C for future C-peptide assay.

**Microcapsule explantation.** Capsules were removed 49 (n = 3), 105 (n = 3), and 210 days (n = 3) after transplantation. Under anesthesia, a 2-cm incision was made in the abdomen, and capsules were explanted by repeated peritoneal lavages with warmed Krebs-Ringer buffer. The abdomen was sutured, and the animals checked daily for blood glucose concentration. Explanted capsules were evaluated microscopically for fibrotic overgrowth and subjected to dithizone staining (Sigma). The capacity to secrete insulin of the recovered encapsulated islets was evaluated immediately after removal by perfusion.

**Splenocyte isolation and transfer to NOD/SCID mice.** After explantation of the microcapsules and return to hyperglycemia, spontaneous diabetic NOD mice were killed; the spleens were removed and placed in RPMI-1640, supplemented with 10% fetal calf serum, penicillin-streptomycin (100 units/ml and 100 μg/ml, respectively), glutamine (2 mmol/l), nonessential amino acids (5 mmol/l; Life Technologies) and β-2-mercaptoethanol (0.5 mmol/l; Sigma). The spleens were dissociated between the frosted ends of two microscope slides, and erythrocytes were lysed in ammonium chloride (0.17 mol/l) for 10 min at room temperature. Splenocytes, the number of which was determined using a hemocytometer, were injected to male NOD/SCID mice (6 to 8 weeks old; Taconic Farms) via the retro-orbital sinus. Blood glucose levels were then checked daily.

**Splenocyte-mixed islet co-culture.** Splenocytes, from nondiabetic male NOD mice, were cultured for 4 days in flat-bottom 96-well plates at a density of 10^6/well with 100 nonencapsulated or encapsulated islets in a final volume of 250 μl. For the final 18 h, H-hymidine (1 μCi/ml; NEN Life Science Products, Boston, MA) was added to the media at a concentration of 1 μCi/well. Cells were then harvested onto a filter and 3H-thymidine incorporation was determined. Each experiment was carried out in triplicate.

**TABLE 2**

Survival results for nonencapsulated islets transplanted under the kidney capsules and encapsulated islets transplanted into the peritoneal cavity

<table>
<thead>
<tr>
<th>Transplanted animal</th>
<th>Islet donors</th>
<th>n</th>
<th>Treatment</th>
<th>Survival (days)</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD</td>
<td>NOD</td>
<td>4</td>
<td>None</td>
<td>4, 7, 2, 2</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

*Time of explantation of encapsulated islets; †time of the death of the mice by old age.
Culture of nonencapsulated and encapsulated islets in the presence of cytokines. Islets were cultured in the presence of cytokines to investigate the potential protective role of the membrane. One hundred nonencapsulated or encapsulated islets were cultured for 3 days in a 24-well plate in 500 μL RPMI-1640 supplemented as described previously in the presence or absence of rat interleukin (IL)-1β (20 U/ml; R & D Systems) and mouse γ-interferon (IFN-γ) (200 U/ml; R & D Systems). The effect of cytokines on islets was investigated by assessment of insulin secretion from perifused islets and by determination of the insulin and DNA content.

Assessment of insulin secretion by perifusion. The ability of free or encapsulated islets to secrete insulin in response to glucose was assessed by perifusion at 37°C in RPMI-1640 supplemented with 10% newborn calf serum with a flow rate of 0.5 ml/min. One hundred handpicked islets were loaded into a chamber (Swinnex 13; Millipore, Bedford, MA) and perifused for 1 h with 2.8 mmol/l glucose followed by 30 min with 16.7 mmol/l glucose and then 50 min at 2.8 mmol/l glucose. Insulin concentrations were measured by RIA and normalized per IE.

Statistical analysis. Data are expressed as means ± SE. Student’s t test for paired data was used to compare data for the IVGTTs, and Student’s t test for unpaired data was used for the in vitro culture. The Mann-Whitney U test was used to analyze perifusion results.

RESULTS

High-M alginate crosslinked with barium is biocompatible. We tested empty capsules placed into NOD or BALB/c mice using two different alginites, two different crosslinking agents, and PLL coating. When removed 30 days later, the biocompatibility of empty capsules was evaluated by determining the retrieval rate, cellular overgrowth on the surface, and capsular breakage (Table 1). With capsules made of high-guluronic acid (high-G) alginate, the retrieval percentage was lower than with capsules made of high-M alginate. PLL coating provoked a clear increase in the number of overgrown and broken capsules. For the high-M alginate, the use of BaCl2 provoked less fibrotic reaction compared to CaCl2-crosslinked capsules.

Encapsulation prolongs islet survival. After transplantation of nonencapsulated NOD islets under the kidney capsule of diabetic NOD mice, normoglycemia was lost at 4 ± 1 days, whereas the survival of nonencapsulated B6AF1 islets was 11 ± 1 and 30 ± 2 days in NOD and BALB/c mice, respectively (Table 2). Encapsulation of NOD islets maintained normoglycemia for >340 days in diabetic NOD mice (Fig. 1A). Likewise, encapsulated B6AF1 islets in spontaneously diabetic NOD (Fig. 1B) and in STZ-induced diabetic BALB/c mice (Fig. 1C) were successful for >350 days. Exploration of islet-containing capsules on days 49, 105, and 210 was immediately followed by reappearance of hyperglycemia. No rejection was observed before capsule removal in mice of any of the groups transplanted with encapsulated islets (Table 2). During IVGTTs, the blood glucose profiles of mice transplanted with encapsulated islets were similar to those of nondiabetic mice (Fig. 2), with no significant difference in the K values (in normal NOD mice, K = 6.5 ± 0.7; in diabetic NOD mice transplanted with encapsulated B6AF1 islets, K = 5.6 ± 0.9, 4.3 ± 0.8, and 7.0 ± 0.7 at 49, 105, and 210 days, respectively). While the peak C-peptide response of the controls was seen at 10 min, it was delayed to 30 min in mice with encapsulated islets. It is noteworthy that a significant increase of C-peptide was found in two of the three groups with encapsulated islets at 10 min.

To determine whether the transplanted NOD mice still had islet-specific immunoreactivity, splenocytes were isolated after capsule removal and transferred to NOD/SCID
mice. All NOD/SCID mice receiving the splenocytes developed diabetes within 16–40 days (Table 3), demonstrating that the transplanted NOD mice maintained autoreactive lymphocytes.

**Encapsulated islets are functional and free of cellular overgrowth.** Immediately after capsule removal, encapsulated islets were assessed for insulin secretion in response to glucose. At 49, 105, and 210 days after transplantation, islets responded to glucose stimulation with a 5- to 10-fold increase in insulin secretion (Fig. 3A and B and Table 4), demonstrating that encapsulated islets remained responsive several months after transplantation. Moreover, the retrieved capsules were free of overgrowth and the contained islets were stained red with dithizone, showing the presence of insulin-containing cells in the capsules (Fig. 4A–C).

**FIG. 2. IVGTTs.** Blood glucose profiles and plasma C-peptide levels of NOD mice transplanted with encapsulated NOD islets (A and D) or B6AF1 islets (B and E) and for BALB/c mice transplanted with encapsulated B6AF1 islets (C and F). IVGTTs were performed 49 days (●, n = 12 or 11), 105 days (▲, n = 9 or 8), and 210 days (○, n = 6 or 5) after transplantation. ●, Diabetic mice; ○, normal mice. For the C-peptide profiles, the results at 7, 15, and 30 weeks were similar and, therefore, combined to provide more statistical power. * P < 0.01; a P < 0.05.

**TABLE 3**
Adoptive transfer of diabetes into NOD/SCID mice

<table>
<thead>
<tr>
<th>Splenocytes from NOD mice transplanted with NOD islets</th>
<th>Splenocytes from NOD mice transplanted with B6AF1 islets</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 weeks</td>
<td>15 weeks</td>
</tr>
<tr>
<td>Splenocytes (× 10⁶)</td>
<td>Diabetes appearance (days)</td>
</tr>
<tr>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>16</td>
<td>40</td>
</tr>
<tr>
<td>99</td>
<td>22</td>
</tr>
<tr>
<td>81</td>
<td>17</td>
</tr>
<tr>
<td>72</td>
<td>27</td>
</tr>
<tr>
<td>20</td>
<td>44</td>
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<tr>
<td>80</td>
<td>101</td>
</tr>
<tr>
<td>46</td>
<td>75</td>
</tr>
<tr>
<td>122</td>
<td>16</td>
</tr>
<tr>
<td>89</td>
<td>22</td>
</tr>
<tr>
<td>138</td>
<td>24</td>
</tr>
</tbody>
</table>

Splenocytes were isolated from transplanted NOD mice at the time of capsule removal (49, 105, and 210 days) and transferred to NOD/SCID mice. Each column presents the number of transferred splenocytes and the time of diabetes appearance after transfer for each NOD/SCID mouse.
Cytokines can cross the membrane and damage islet cells. To determine whether capsules protect against cytokine-related islet destruction, B6AF1 islets, encapsulated or nonencapsulated, were cultured in the presence or absence of IL-1β and IFN-γ for 3 days. Insulin secretion assessed by perfusion of nonencapsulated islets exposed to cytokines was reduced compared with controls (Fig. 5A). Insulin secretion from the perfused encapsulated islets was reduced both with and without cytokine exposure, with no significant difference between the two groups (Fig. 5B). The insulin-to-DNA ratio was markedly reduced in both unencapsulated and encapsulated islets after exposure to cytokines (Fig. 6).

Co-culture of encapsulated islets with splenocytes isolated from NOD mice. In the presence of nonencapsulated islets, incorporation of 3H-thymidine by splenocytes was increased 2.8-fold ($P < 0.02$, Fig. 7), whereas no immune response was observed when encapsulated islets were co-cultured with splenocytes.

**DISCUSSION**

We used a simple technique that consists of one step encapsulation with a highly purified high-M alginate crosslinked with barium. These capsules are stable and highly biocompatible in NOD and BALB/c mice, consistent with a previous finding that high-M alginites are as biocompatible as high-G alginites (24). In addition, the high purity of the alginate and the reduction in endotoxin levels probably accounted for much of our success.

We demonstrated that normoglycemia could be achieved in all STZ-induced diabetic and spontaneously diabetic NOD mice transplanted with syngeneic and allogeneic islets enclosed within this barium-alginate membrane (Fig. 1). Most importantly, encapsulated islets were able to reverse diabetes for >350 days. Glucose profiles during IVGTTs performed at 210 days were normal (Fig. 2), with C-peptide responses that were delayed and lower than controls. It is important to note that significant secretion was found only 10 min after the glucose stimulus, which is consistent with our recent report (25) and provides optimism that the dynamics of insulin release from microcapsules are fast enough for clinical situations. The efficacy of the encapsulated islets was confirmed by return to hyperglycemia immediately after the explantation of the encapsulated islets. To date, there have been no failures in any of the three groups. Microscopic examination of removed capsules showed that capsules were free of capsular overgrowth and that the islets contained viable insulin-containing β-cells, as evidenced by dithizone staining.

**TABLE 4**

Insulin secretion of explanted capsules during perfusion with 2.8 mmol/l glucose and at the peak of stimulation

<table>
<thead>
<tr>
<th>Time</th>
<th>NOD → NOD</th>
<th>B6AF1 → NOD</th>
<th>B6AF1 → BALB/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal (pg min⁻¹ IE⁻¹)</td>
<td>Peak (pg min⁻¹ IE⁻¹)</td>
<td>Basal (pg min⁻¹ IE⁻¹)</td>
<td>Peak (pg min⁻¹ IE⁻¹)</td>
</tr>
<tr>
<td>7 weeks</td>
<td>4.5</td>
<td>7.3</td>
<td>2.6</td>
</tr>
<tr>
<td>15 weeks</td>
<td>3.0</td>
<td>31</td>
<td>3.5</td>
</tr>
<tr>
<td>30 weeks</td>
<td>ND</td>
<td>ND</td>
<td>2.0</td>
</tr>
<tr>
<td>30 weeks</td>
<td>1.5</td>
<td>21</td>
<td>3.9</td>
</tr>
</tbody>
</table>

ND, not determined.
Moreover, perifusion of explanted capsules revealed good secretory function, even 210 days posttransplantation, with typically 5- to 10-fold increases of glucose-stimulated insulin secretion (Fig. 3 and Table 4).

To understand the mechanisms by which the membrane protects the islets in vivo, we evaluated the effect of cytokines on encapsulated islets. A previous study showed that islets are protected against IL-1β in vitro (26), whereas other reports suggested that cytokines could cross the membrane and affect the function of the encapsulated islets (15, 27). Our results confirm that cytokines can cross the membrane and damage the islet cells contained inside the capsules. Thus, although cytokines have the potential to cause problems for encapsulated islets, the lack of a cellular reaction at the capsular surface suggests that local cytokine production from inflammatory cells is not an issue in this experimental situation.

Further insight into the potential vulnerability of encapsulated islets was tested in a splenocyte-islet co-culture system, with the finding that nonencapsulated islets induced a 2.8-fold increase in lymphocyte proliferation, whereas encapsulated islets had virtually no effect (Fig. 7). These results indicate that capsules prevent communication between islets and splenocytes that might cause lymphocyte activation and that the barium alginate is biocompatible in this setting. Some earlier studies have found a similar lack of lymphocyte activation (24, 28); activation found by others might have been due to contaminants (29). Thus, the combination of biocompatible alginate and shedding of antigens from islets that may be below the detection limit of the immune surveillance system of the recipients probably explains the avoidance of allorejection and autoimmune diabetes recurrence.

In conclusion, these results provide evidence that a simple alginate barrier, without a traditional permselective layer such as PLL or poly-ethylene-glycol, can provide complete protection against allorejection and autoimmu-
Survival of Microencapsulated Islets in NOD Mice

FIG. 6. Insulin-to-DNA ratio after 3 days of culture in the presence or absence of cytokines ($n = 4$, *$P < 0.05$). I, naked islets; C, encapsulated islets; CK, presence of cytokines in the media.

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