Cell Transplantation, Vol. 22, pp. 1137–1146, 2013

0963-6897/13 $90.00 + .00
DOI: http://dx.doi.org/10.3727/096368912X657486
E-ISSN 1555-3892
www.cognizantcommunication.com

The TheraCyte™ Device Protects Against Islet Allograft Rejection in Immunized Hosts

Makiko Kumagai-Braesch,* Stella Jacobson,† Hiroki Mori,* Xiaohui Jia,* Tohru Takahashi,* Annika Wernerson,‡ Malin Flodström-Tullberg,† and Annika Tibell*

*Division of Transplantation Surgery, CLINTEC, Karolinska Institutet, Stockholm, Sweden
†Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska Institutet, Stockholm, Sweden
‡Division of Renal Medicine, CLINTEC, Karolinska Institutet, Stockholm, Sweden

Clinically, many candidates for islet transplantation are already immunized, which increases their risk of graft rejection. Encapsulation of pancreatic islets using the TheraCyte™ device has been shown to protect against allograft rejection in nonimmunized recipients. However, the capacity of the TheraCyte™ device to prevent rejection in immunized recipients has not yet been studied. In this study, the protective capacity of the TheraCyte™ device was evaluated in an allogeneic rat model. Lewis rats were used as islet donors, and nonimmunized (control) and alloimmunized, diabetic Wistar–Furth (WF) rats were used as recipients. Graft survival was shorter in immunized recipients than in nonimmunized recipients (mean survival, 5.3 ± 2.7 and 9.3 ± 1.6 days, respectively, $p < 0.01$) when nonencapsulated islets were transplanted under the kidney capsule. When islets were transplanted into the TheraCyte™ device, graft function was maintained during the 6-month study period in both immunized and nonimmunized rats. In oral glucose tolerance tests performed at 1 month after transplantation, both groups had similar insulin and blood glucose levels indicating similar metabolic functions. Volume densities and absolute volumes of tissue inside the devices 6 months after transplantation were also comparable between the two groups, indicating that both groups maintained similar amounts of endocrine tissue. A higher number of IFN-γ-producing $CD8^+$ T-cells were detected in immunized WF rats compared to control WF rats transplanted with encapsulated islets. This suggests that donor-specific alloreactivity in recipient rats was sustained throughout the study period. This study suggests that the TheraCyte™ device protects islet allografts also in immunized recipients. Our results further highlight the potential for using macroencapsulation to avoid immunosuppressive therapy in clinical islet transplantation.

Key words: Islet transplantation; Immunized recipients; TheraCyte™ device; Macroencapsulation; Alloantibodies; IFN-γ ELISpot

INTRODUCTION

Islet transplantation can restore blood glucose metabolism in type 1 diabetic patients. However, the side effects associated with the chronic immunosuppressive therapy outweighs the benefits of islet transplantation for many patients. Furthermore, long-term islet graft function is still unsatisfactory (16). Currently, islet transplantation is therefore recommended for only a small portion of type 1 diabetic patients.

A promising method to avoid the need for long-term immunosuppression is encapsulation of the islet graft inside an immunoprotective membrane. The TheraCyte™ device has an outer membrane that facilitates neovascularization and an inner immunoprotective membrane. In our rodent model, the curative dose of transplanted macroencapsulated islets was found to be 10 times higher than the curative dose of nonencapsulated islets, mainly due to the lack of sufficient vascularization of the freshly implanted device. To circumvent this issue, we had previously evaluated the preimplantation of empty TheraCyte™ devices 3 months prior to islet transplantation and have shown that this approach dramatically reduces the number of islets required to cure (19).

The TheraCyte™ device has been shown to protect allografts from rejection in nonimmunized rodent and human recipients (1,22) and also protects the recipient from sensitization (i.e., production of alloreactive antibodies) (20,22). Furthermore, the immunoprotective capacity of the device has been shown to be effective in autoimmune animal models (21,25). However, xenogeneic grafts are

Received November 11, 2010; final acceptance June 3, 2012; Online prepub date: October 3, 2012.
Address correspondence to Makiko Kumagai-Braesch, Division of Transplantation Surgery, CLINTEC, Karolinska Institutet, Karolinska University Hospital Huddinge F82, 141 86, Stockholm, Sweden. Tel: +46 8 585 89520; Fax: +46 8 711 5202; E-mail: makiko.kumagai-braesch@ki.se

not protected by this device, instead leaking xenoantigens cause a strong inflammatory reaction around the implant. As the inner membrane is permeable to antibodies and inflammatory factors, this reaction leads to destruction of the encapsulated xenograft (2,10–12).

We have not found any data on the immunoprotective capacity of the TheraCyte™ device in alloimmunized recipients. This is clinically relevant because many patients who would benefit from islet transplantation have already been immunized as a result of pregnancy, transfusion, or previous transplantation. Furthermore, it has previously been shown that alloimmunization may have deleterious effects on a subsequent islet graft [reviewed in Harlan et al. (5)].

Here, we evaluate the immunoprotective capacity of the TheraCyte™ device in immunized recipients using a fully major histocompatibility complex (MHC)-mismatched rat islet transplantation model.

MATERIALS AND METHODS

Animals

Wistar–Furth (WF) (Scanbur, BK AB, Sollentuna, Sweden) and Lewis rats (Charles River, Sulzfeld, Germany) were maintained at Karolinska Institutet, Stockholm, Sweden. All animal experiments were approved by the local ethics committee and were conducted in accordance with institutional guidelines for animal care and use.

Immunization of Recipients

Female WF rats (200 g) were immunized via the transplantation of 1,000 Lewis islets under the left kidney capsule ($n = 17$) or by intraperitoneal (IP) injection of 2×10^8 irradiated Lewis splenocytes ($n = 13$). Four weeks after, immunization was confirmed by the presence of antidonor antibodies (IgG isotype) in recipient serum (see "Detection of Anti-Lewis Rat Antibodies" below).

In the group transplanted with nonencapsulated islets under the kidney capsule, eight rats were immunized by islet transplantation and four rats were immunized by splenocyte injection. In the group receiving macroencapsulated islets, five rats were immunized by islet transplantation and two rats were immunized by splenocyte injection. However, in one of the rats that were immunized by splenocyte injection and transplanted with macroencapsulated islets, endogenous β-cell function had recovered 6 months after streptozotocin (STZ) injection (see "Posttransplant Management"), and it was therefore removed from the study. Thus, six preimmunized rats that were transplanted with macroencapsulated islets were used for this study.

Pancreatic Islet Isolation

Islets from male Lewis rats (300 g) were isolated as previously described (18). Briefly, rat pancreases were digested using 0.7 mg/ml collagenase P (Roche Diagnostics GmbH, Mannheim, Germany), and the islets were purified using discontinuous density gradient centrifugation (Histopaque-1119, Histopaque-1077; Sigma, Stockholm, Sweden). Isolated islets were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, BRL, Life Technology Ltd., Paisley, Scotland, UK) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100 mg/L streptomycin (all provided by Gibco) at 37°C in a humidified (5% CO_2, 95% air) atmosphere. Islets were cultured for 20 h prior to islet transplantation.

Implantation of the Theracyte™ Device, Induction of Diabetes, and Islet Transplantation

Empty TheraCyte™ devices (TheraCyte, Inc., Laguna Hills, CA, USA) were implanted under the skin of nondiabetic WF rats (19). A 1-cm transverse incision was made on the back of each rat. A subcutaneous pocket was created by blunt dissection, and the sealed device was implanted. After 3 months, diabetes was induced by a single intravenous injection of streptozotocin (Sigma; 50 mg/kg body weight) 4 days prior to transplantation. Animals were considered diabetic if blood glucose exceeded 20 mmol/L (>360 mg/dl) for at least two consecutive days. A total of 1,000 islets were handpicked and washed once with Hank's balanced salt solution (HBSS, Karolinska University Hospital, Stockholm, Sweden) and then loaded into the preimplanted TheraCyte™ devices in the diabetic WF rats using a neonatal Venflon (BD, Helsingborg, Sweden) attached to a Hamilton syringe (Hamilton Company, Reno, NV, USA). After loading, the port was sealed with glue (Silastic® medical adhesive silicone type A; Dow Corning Corporation, Midland, MI, USA) to avoid islet leaking and the growth of host tissue into the lumen of the device. For this study, the internal volume of the device used was 40 µl.

For islet transplantation under the kidney capsule, 1,000 Lewis rat islets were picked and washed once with HBSS and then packed into a 22-gauge Venflon™ (BD). The islets were placed under the kidney capsule of diabetic WF rats as previously described (18). For immunization, islets were transplanted under the left kidney capsule, and when the rats were retransplanted to cure diabetes, islets were placed under the right kidney capsule.

Posttransplant Management

The nonfasting blood glucose levels and weights of the animals were measured daily for the first week. Thereafter, rats with blood glucose levels of <10 mmol/L for 2 consecutive days were assessed twice a week. Rats with hyperglycemia were monitored daily. Blood glucose levels of >20 mmol/L for 2 consecutive days were taken as an indication of graft rejection, and these

rats were euthanized. Euglycaemic animals were followed for 6 months. Subsequently, graftectomy under enflurane anesthesia was performed, and blood glucose levels were followed for another 2–3 days in order to confirm the recurrence of diabetes.

Oral Glucose Tolerance Test

An oral glucose tolerance test (OGTT) was performed 1 month after transplantation in rats cured of diabetes. A total of 1.5 g glucose/kg bodyweight (BDH-Prolabo, VWR International AB, Stockholm, Sweden) was administered orally to rats after 16 h of fasting, and blood glucose levels were measured at 0, 15, 30, 45, 60, 90, and 120 min after glucose challenge. Insulin levels in plasma were measured at 0, 30, 60, and 120 min using a rat insulin ELISA kit (Mercodia, Uppsala, Sweden). Healthy male Lewis rats and female WF rats were used as controls.

Histological Analysis

Methods for calculating the total volumes of graft tissue and fractional volumes of different types of tissues within the TheraCyte™ device have been described previously (15,19). Briefly, explanted devices were fixed in 4% phosphate-buffered formalin (Solveco AB, Rosersberg, Sweden) and then cut in four pieces and embedded two and two in paraffin. The blocks were subsequently sectioned in 5-µm-thick serial sections throughout the entire block. Sections at 180-µm intervals were stained with hematoxylin and eosin (H&E; Histloab Products AB, Göteborg, Sweden).

To quantify the total volume of tissue inside the device, a Leica DMR XA microscope was utilized to measure the graft tissue area using computerized acquired image analysis (Leica QWin Software, Kista, Sweden). The absolute volume of graft tissue within a device was estimated by multiplying the tissue area of each stained section by the distance to the next stained section and then calculating the sum of all of these volumes in one block.

To estimate fractional volumes, that is, volume densities of viable fibroblasts and endocrine cells, conventional stereological principles were used. Three levels of H&E sections from the same device were photographed at 10× magnification and printed on paper copies at a final magnification of 220. Point counting of fibrous and endocrine tissues were performed using a square lattice (1 cm), made on a transparent overhead sheet, randomly placed over the print out, and then the percentage of each tissue was calculated. The absolute volume of each tissue type was then calculated by multiplying the volume density (%) by the total tissue volume (24). On the H&E sections, viable endocrine cells have round nuclei, and a homogenous shape and nonendocrine tissue with elongated nuclei represent fibroblasts.

Immunohistochemical staining was performed for the detection of insulin-containing cells (NCL-INSULIN Visionbiosystems, Novocastra, Leica Biosystems Newcastle Ltd., Newcastle Upon Tyne, UK). Sections were stained by semiautomated methods (the Bond-maX Max; Leica Microsystems) using routine techniques used at the Department of Pathology, Karolinska University Hospital Huddinge, Sweden. All insulin-stained sections were carefully compared to adjacent sections stained by H&E, confirming that the majority of the endocrine cells were insulin positive.

In Vitro Examination of the Efficiency of the Immunization Protocols

Detection of Antidonor Antibodies. Four weeks postimmunization, serum was harvested from the top of the tail of WF rats and the presence of antibodies against Lewis rat splenocytes depleted of B-cells using anti-rat κ-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Ten microliters of WF serum and 100 µl of Lewis non-B-cell splenocytes suspension (10^6 splenocytes) were incubated at 4°C for 30 min, labeled with both fluorescein isothiocyanate (FITC) anti-rat IgG and anti-rat IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) or IgG subclass-specific antibodies, that is, anti-rat IgG1, IgG2a, and IgG2b (clones R1-3G1, R2a-21B2, and R2B-7C3, respectively; eBioscience, San Diego, CA, USA) and analyzed by flow cytometry (FACScan, BD). An IgG antibody mean fluorescence intensity greater than 10 channels more than that observed with nonimmunized WF rat serum was considered positive for alloantibodies. None of the nonimmunized rats had anti-Lewis antibodies (IgG isotype). Therefore, the presence of anti-Lewis antibodies was used as a marker to indicate successful immunization.

Proliferation Tests. To confirm the presence of memory T-cells 4 weeks after immunization, spleens from WF rats were harvested after euthanasia. Splenocytes from nonimmunized WF rats were used as controls. $CD4^+$ and $CD8^+$ T-cells were isolated from splenocytes using anti-rat CD4 or CD8 beads (CD4 for negative selection and CD8 for positive selection, Miltenyi Biotec). Lewis splenocytes (stimulator cells) were isolated and exposed to 20-Gy radiation (Mølgaad Medical Aps, Horsholm, Denmark). Subsequently, 10^5 responder cells/well and 4×10^5 stimulator cells/well were mixed to a final volume of 200 µl in RPMI 1640 supplemented with 10% FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol (all provided by Gibco). The proliferation test was performed in triplicate in U-bottomed, 96-well tissue culture plates (BD). Cells were incubated at 37°C in humidified air containing 5% CO_2. Each culture was labeled with 2 µCi [^{3}H]thymidine (Amersham Biosciences, Little Chalfont,

Buckinghamshire, UK) approximately 20 h prior to harvest. [^{3}H]Thymidine incorporation was measured using a beta counter (Wallac Sverige AB, Upplands Vasby, Sweden).

Stimulation index (SI) was calculated by dividing the [^{3}H]thymidine incorporation with stimulator cells by the [^{3}H]thymidine incorporation in control wells without stimulator cells.

IFN-γ ELISpot Assay. Interferon-γ (IFN-γ) ELISpot assays were used to detect reactive T-cells from each donor (13,14,23). WF splenocytes harvested 4 weeks after immunization or splenocytes harvested from four islet transplanted groups at the time of euthanasia were used as responder cells. Cells from the islet transplanted groups were immediately frozen in 10% dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany), 40% FBS in culture medium, and kept in liquid nitrogen until analysis. CD4$^+$ and CD8$^+$ T-cells were isolated from WF splenocytes as described above. Fresh Lewis rat splenocytes were isolated and used as stimulators. All stimulator cells were exposed to 20-Gy radiation.

Pretreated 96-well ELISpot plates (MSIP-TW) and the anti-rat IFN-γ ELISpot kit were a gift from Mabtech AB (Nacka, Sweden). Pretreated ELISpot plates did not need to be prewetted with ethanol, and capture antibody (10 μg/ml, 100 μl/well) was added directly to the plates and incubated overnight at 4°C. Plates were then washed six times with phosphate-buffered saline (PBS; Karolinska University Hospital). In a final volume of 200 μl/well culture medium, responder cells were cocultured with 4×10^5 Lewis rat splenocytes/well. The plates were incubated at 37°C in a humidified (5% CO_2, 95% air) atmosphere for 18 h. The ELISpot assay was then performed according to the manufacturer's guidelines. The quantification of spot-forming units and cytokine activity was measured by the ELISpot counter software, version 3.5 (AID, Strasburg, Germany). *Cytokine activity* was included in the manufacturer's software and signifies a relative quantification of the level of cytokine.

Statistics

Statistical analyses were performed using GraphPad Prism software, version 5 (GraphPad Software, Inc., San Diego, CA, USA). Differences in IFN-γ production and OGTT area under the curve were analyzed by one-way ANOVA, followed by Dunn's posttest. Differences between the two groups in proliferation tests, IFN-γ production, and volume densities were analyzed by the nonparametric Mann–Whitney test, but the data are represented by mean ± SD for clarity. Survival was analyzed by log-rank (Mantel–Cox) test with an adjustment for multiple comparisons by Bonferroni posttest. A value of $p<0.05$ was considered statistically significant.

RESULTS

Immunized Rats Produce Antidonor Antibodies

Immunization was performed by either Lewis rat islet transplantation or splenocyte injection. To confirm immunization, the presence of antibodies in WF rat serum against Lewis rat antigens (donor-specific antibodies) was assessed by flow cytometry 1 month after immunization. All rats that were immunized by islet transplantation, and 11 of 13 rats immunized by IP injection of irradiated splenocytes had detectable levels of IgG against Lewis rat splenocytes in serum. Most of the immunized rats had IgG1 and IgG2a antibodies, and some had IgG2b (data not shown). Eight of 17 rats immunized by transplantation and 4 of 11 rats received irradiated splenocytes had detectable levels of antidonor antibodies of both IgG and IgM isotypes, but the IgM levels were generally lower. Representative FACS histograms of the presence of IgG and IgM anti-bodies in an immunized WF rat are shown in Figure 1. One of 30 non-immunized control rats had alloreactive IgM, but none had IgG antibodies (data not shown).

Donor-specific antibodies were also examined in the serum of nonimmunized and immunized WF rats transplanted with encapsulated islets. Nonimmunized recipients ($n=6$) did not produce donor-specific antibodies during the 6-month observation period. Four of six immunized recipient rats maintained detectable levels of donor-specific antibodies. However, the antibody titers were slightly reduced at the end of the 6-month observation period.

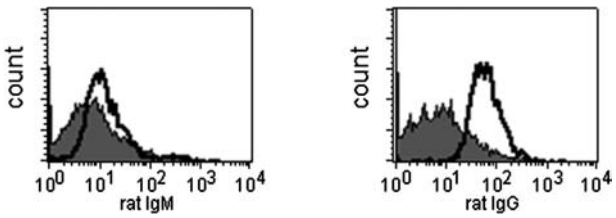

Figure 1. Immunized WF rats produce alloantibodies against Lewis rat splenocytes. The presence of allospecific IgM and IgG antibodies in serum from immunized or nonimmunized Wistar–Furth (WF) rats were examined using Lewis spleen cells (depleted of B-cells) as target cells. Representative histograms of IgG and IgM antibodies levels in serum from one immunized WF rat (bold line), which was immunized by islet transplantation and subsequently used for macroencapsulated islet transplantation, and one control rat (filled curve) are shown. High levels of IgG were detected in immunized rats, but IgM levels were not clearly increased.

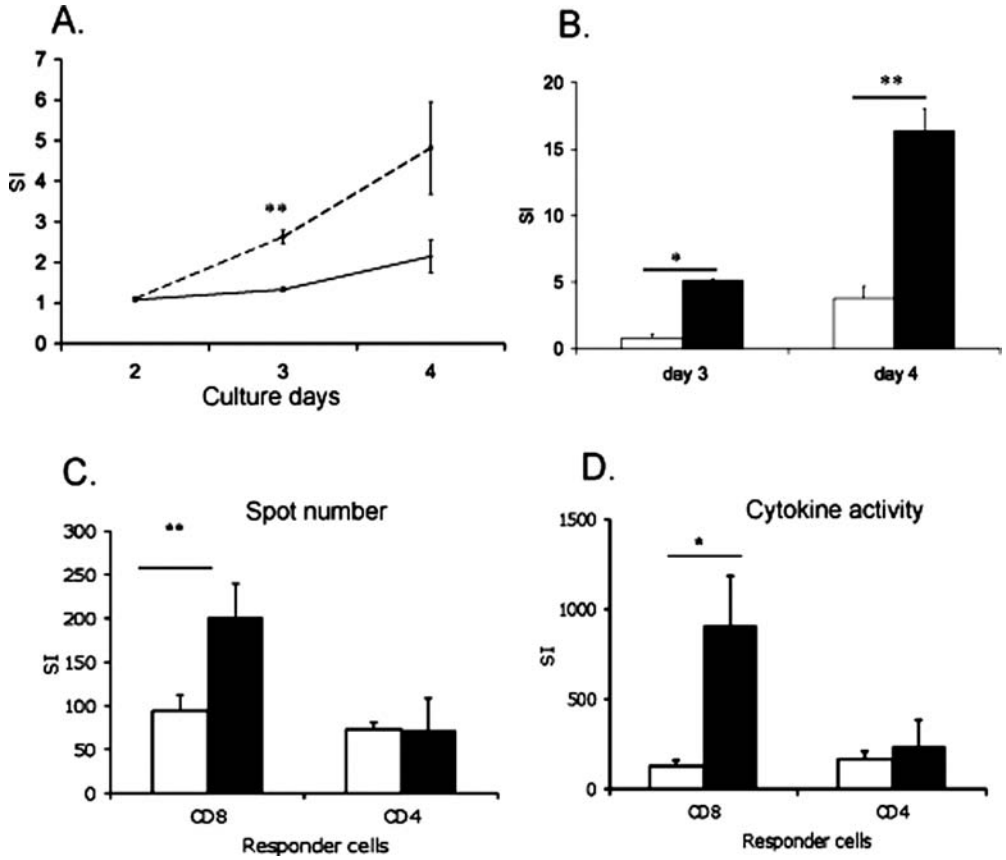

Figure 2. Increased cell proliferation and IFN-γ production by CD8⁺ T-cells from immunized WF rats after stimulation with Lewis splenocytes. (A) Kinetics of proliferative responses of WF splenocytes against Lewis splenocytes are shown. Broken line represents splenocytes from immunized WF rats. Solid line indicates splenocytes from nonimmunized control WF rats. The mean stimulation index (SI; $n=3$ in each group) are shown. (B) SI of CD8⁺ T-cell proliferation after 3 and 4 days of incubation with Lewis splenocytes. The results are shown as the mean ± SD. Open bars indicate nonimmunized control group ($n=3$), and filled bars immunized groups ($n=4$). (C, D) Results from interferon-γ (IFN-γ) ELISpot assays. In this experiment, freshly prepared responder cells were used at a concentration of 10^5 cells/well. (C) SI of spot number and (D) cytokine activity are shown. The results are presented as the means ± SD. Open bars indicate the nonimmunized control group ($n=3$), and filled bars the immunized group ($n=4$). *$p<0.05$; **$p<0.01$.

In Vitro Characterization of T-Cell Responses After Immunization

The response of recipient WF splenocytes against donor Lewis splenocytes was examined in proliferation tests and IFN-γ ELISpot assays. The immunized group showed a higher proliferative rate than nonimmunized controls. The SI for the immunized group was significantly higher than that of the nonimmunized group after 3 days (Fig. 2A). Purified CD8⁺ T-cells from immunized rats showed both a higher proliferative response (Fig. 2B) and a higher level of IFN-γ production compared to nonimmunized control (Fig. 2C, D). Proliferation and IFN-γ production by purified CD4⁺ T-cells of immunized rats in the response to Lewis rat splenocytes was not significantly different from that in nonimmunized controls. Similar results were observed in animals immunized by Lewis rat islets or splenocyte injections. The results show that, after immunization, CD8⁺ T-cell activity against donor splenocytes was significantly increased.

Encapsulated Islets Are Protected From Rejection Also in Immunized Recipients

As expected, both nonimmunized and immunized rats that were transplanted with islets under the kidney capsules rejected the grafts. The immunized rats rejected the grafts more rapidly than nonimmunized rats (mean survival 5.3 ± 2.7 and 9.3 ± 1.6 days, respectively, $p<0.01$).

In contrast, all nonimmunized rats that received macroencapsulated islets remained euglycemic throughout the 6-month study period (Fig. 3). Interestingly, all immunized rats transplanted with encapsulated islets were also protected from allograft rejection.

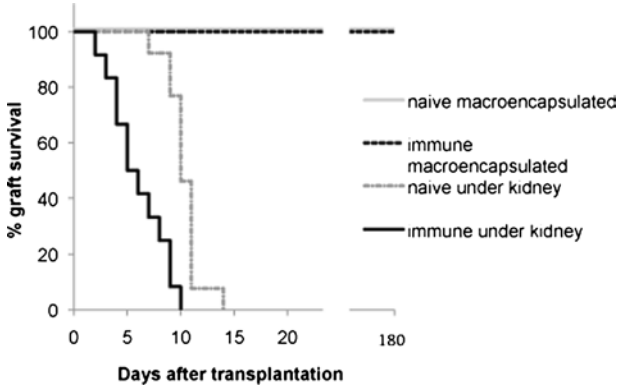

Figure 3. Encapsulated Lewis islets transplanted to immunized WF rats are protected from allograft rejection. Islet graft survival after transplantation is shown. The immunized WF rats that received islets under the kidney capsules (black solid line, $n=12$) rejected the grafts more quickly than nonimmunized rats (gray broken line, $n=13$) (mean survival 5.3 ± 2.7 and 9.3 ± 1.6 days, respectively, $p<0.01$). Nonimmunized (gray solid line, $n=6$) and immunized (black broken line, $n=6$) WF rats were transplanted with Lewis islets into the TheraCyte™ device. Encapsulated islet grafts also survived for at least 6 months in immunized recipients.

All rats that were cured of diabetes underwent graftectomy after 6 months. In these rats, hyperglycemina subsequently reoccurred within 24 h. The rats were sacrificed 1–2 days later. Recipient pancreases harvested at the time of euthanasia contained only a few islets and had an almost undetectable insulin stain compared to healthy controls.

Nonimmunized and Immunized Rats Transplanted With Islets Into the TheraCyte™ Device Respond to OGTT With Similar Kinetics

To investigate the metabolic function in immunized and nonimmunized WF rats transplanted with encapsulated islets, the response to OGTTs was assessed 1 month posttransplantation (Fig. 4).

The kinetics of blood glucose and plasma insulin levels were similar in the two transplanted groups, indicating that immunization status did not affect the functional capacity of the transplanted islets. However, when compared to healthy controls, individual variations in blood glucose and plasma insulin levels were higher in rats that received the transplantation. The blood glucose levels of both nonimmunized and immunized rats transplanted with encapsulated islets were higher compared to the control rats. This difference was only statistically significant for immunized rats compared to Lewis control rats ($p<0.05$) (Fig. 4A).

Plasma insulin levels, measured at 0, 30, 60, and 120 min after glucose stimulation, tended to be higher in the transplantation groups compared to the healthy controls, but these differences were not statistically significant (Fig. 4B).

Immunization Status Did Not Affect Volumes of Endocrine and Fibrotic Tissues in the TheraCyte™ Devices

The volume of endocrine and fibrotic tissue inside the explanted devices was determined by quantitative histological analysis. No differences in the total tissue volume or the absolute volume of endocrine and fibrotic tissues were detected between nonimmunized and immunized recipients. This indicates that the immunization of the recipient rats did not affect the survival of the grafted tissue when protected by the TheraCyte™ device (Table 1).

Representative insulin-stained sections from encapsulated grafts 6 months after transplantation from both immunized and nonimmunized recipients are shown in Figure 5A–D, respectively. The volume of endocrine tissue roughly corresponded to the β-cell volume because most of the endocrine tissue was insulin positive (Fig. 5A, C). No inflammatory cell infiltration was observed in close proximity to the outer membranes of the TheraCyte™ device in any of the groups. However, significant fibrosis had developed inside the devices in both groups (Fig. 5B, D and Table 1).

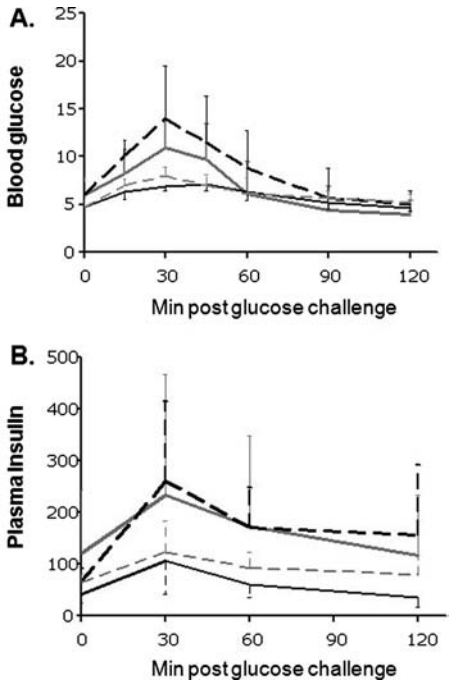

Figure 4. No significant difference in response to an oral glucose challenge 1 month after transplantation. The figure shows blood glucose and plasma insulin levels during oral glucose tolerance test (OGTT) with nonimmunized (gray line, $n=6$) and immunized (black broken line, $n=6$) WF rats transplanted with encapsulated islets, healthy Lewis (black line, $n=15$) and WF control rats (gray broken line, $n=10$). Differences in (A) blood glucose and (B) plasma insulin levels were compared between the groups. The results are presented as the mean ± SD.

Table 1. Absolute Volumes (mm^3) of Endocrine and Fibrotic Tissues Within the TheraCyte™ Devices at 6 Months Posttransplantation

	Nonimmunized Recipients	Immunized Recipients
Total tissue volume	5.10±2.25	4.68±1.80
Endocrine tissue	0.88±0.45	0.72±0.18
Fibrotic tissue	3.93±1.55	3.56±1.55

Values are presented as the means ± SD. For nonimmunized recipients, $n=4$. For immunized recipients, $n=6$. Total tissue volume, sum of endocrine and fibrotic tissue volumes are shown. No significant differences were seen.

$CD8^+$ T-Cells From Immunized Rats That Received Islets in the TheraCyte™ Device Produce IFN-γ in Response to Donor Cells

To verify that the allospecific response of immunized recipients was maintained at 6 months after encapsulated islet transplantation, IFN-γ production by recipient $CD8^+$ T-cells in response to donor splenocytes was evaluated with ELISpot assays (Fig. 6A). $CD8^+$ T-cells from immunized WF rats transplanted with islets in the TheraCyte™ device produced significantly more IFN-γ (SI of spot number was 27±10, corresponding to a cytokine activity of 86±44) compared to naive WF rats (SI spots 6±3, cytokine activity 7±3; $p<0.05$) and nonimmunized WF rats transplanted with encapsulated islets (SI spots 5±2, cytokine activity 7±4; $p<0.05$).

IFN-γ production by $CD8^+$ T-cells from nonencapsulated islet transplantation groups was also evaluated (Fig. 6B). Cells in these groups were harvested at the time of rejection and therefore cannot be directly compared with cells in the encapsulated groups. Immunized rats transplanted with nonencapsulated islets under the kidney capsule showed a higher production of IFN-γ than nonimmunized recipients (SI spots: 110±60 vs. 22±11; SI cytokine activity: 445±232 vs. 58±47, respectively).

A typical example of an ELISpot image from each group is shown below the figures. Taken together, these results indicate that sensitization of the recipient rats was sustained throughout the study period.

DISCUSSION

Our studies show, for the first time, that the TheraCyte™ device is capable of protecting allogeneic islet grafts in immunized recipients. Functional and morphological studies confirmed the protection of the encapsulated islets in the presence of allospecific antibodies and alloreactive $CD8^+$ T-cells for at least 6 months after transplantation. When immunized recipients were transplanted with nonencapsulated islets under the kidney capsule, the mean survival time was 5 days, whereas naive recipients of nonencapsulated islets were rejected within 2 weeks.

To our knowledge, previous studies using macroencapsulation devices have not evaluated their immunoprotective capacity in alloimmunized recipients, and we have not found any studies of microencapsulated alloislet transplantation in presensitized recipients. However, posttransplant sensitization has been described in microencapsulated transplantation models, and it is thought to be related to incomplete encapsulation of parts of the islet graft (8,17). Several studies indicate that microencapsulation continues to provide protection of islets that are completely covered. Sensitization and secondary inflammation could contribute to the development of fibrosis around the microencapsulated islets.

Clinically, islet transplantation to immunized recipients is a common challenge. Often, islet graft recipients need repeated transplantations to obtain euglycemia. Furthermore, graft survival time after islet transplantation is limited, and some patients must undergo a new transplantation after only a few years. Thus, many patients have developed alloreactive antibodies (3,9). Our results indicate that the TheraCyte™ device may also be useful in the patients with alloantibodies as a result of either previous islet transplantation or other causes.

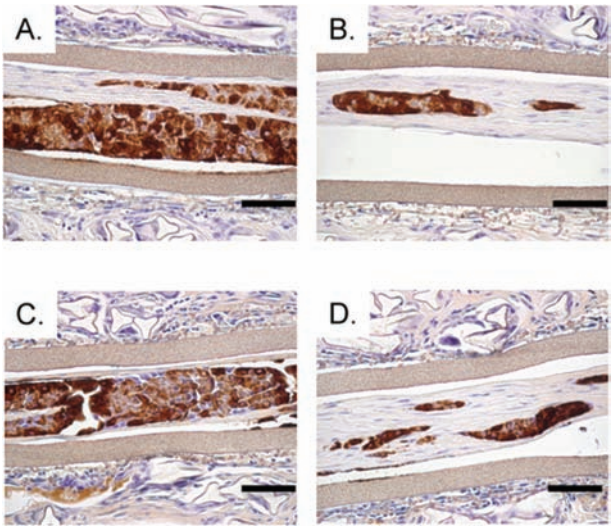

Figure 5. Similar survival of encapsulated islet grafts in nonimmunized and immunized WF rats at 6 months after transplantation. (A, B) Representative insulin-stained sections from an encapsulated graft taken from an immunized recipient 6 months after transplantation. (C, D) Representative insulin staining of sections from a nonimmunized recipient. Brown staining indicates insulin positive cells. The morphology of the insulin negative cells, with their elongated nuclei, indicate that they represent fibroblasts. Fibrotic tissue inside the device was observed in both groups (most prominent in B, D). Scale bars: 50 μm. The results from the quantitative histological analysis are shown in Table 1.

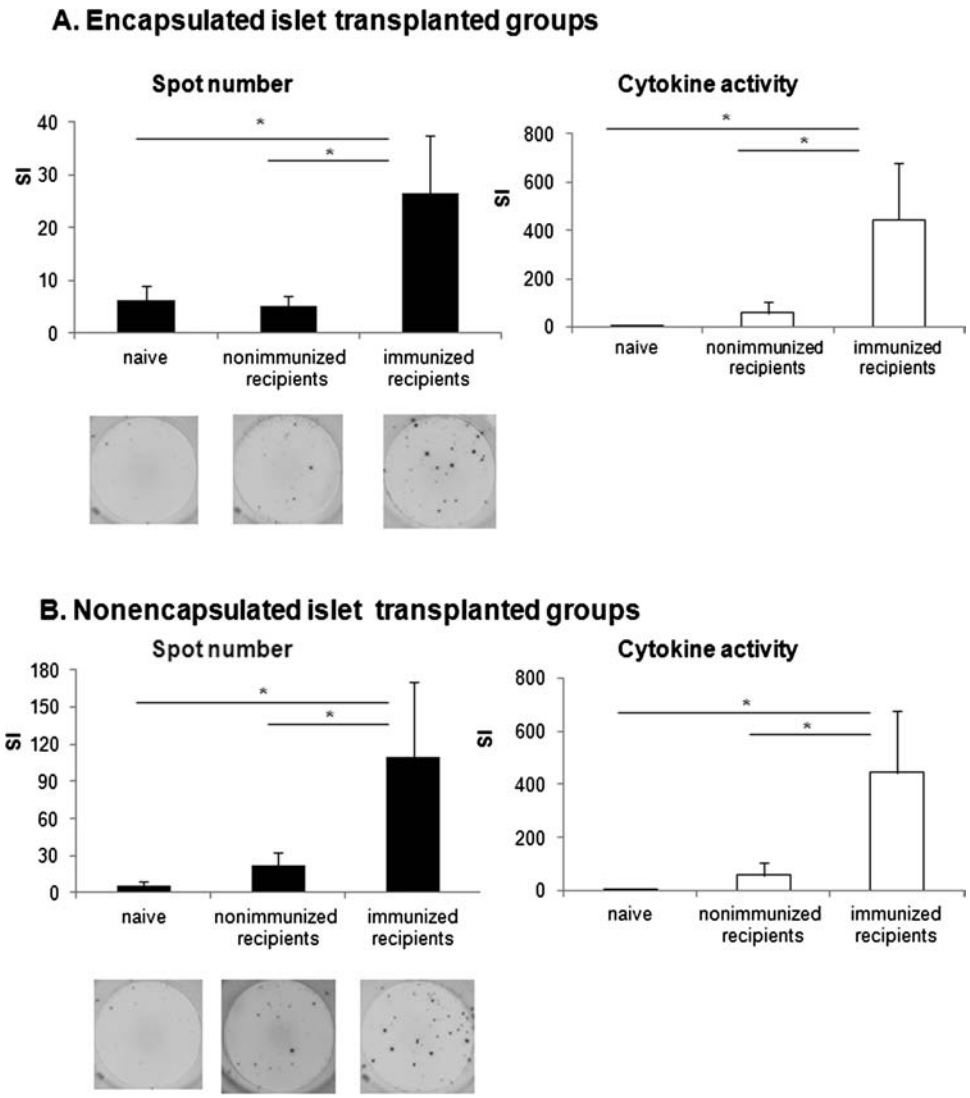

Figure 6. Alloreactive CD8+ T-cells are detected in immunized WF rats transplanted with islets into the TheraCyte™ device 6 months after transplantation. To further evaluate the allospecific response, IFN-γ production by CD8+ T-cells (2.5×10^4/well) from cryopreserved splenocytes were analyzed. Bar graphs show SI of the number of spots (left figures, black bars) and cytokine activity (right figures, open bars). (A) IFN-γ production by CD8+ T-cells 6 months after encapsulated islet transplantation. Splenocytes were harvested 6 months after transplantation. Naive rats that did not receive a transplant and did not have diabetes were used as a negative control. SI of naive WF rats ($n=10$), nonimmunized WF rats transplanted with islets in the TheraCyte™ device ($n=6$), and immunized rats transplanted with islets in the device ($n=6$) are compared. Representative images from ELISpot wells are shown under each figure. (B) IFN-γ production by CD8+ T-cells after rejection of nonencapsulated islets. Cells were harvested at the time of rejection. Naive WF rats, nonimmunized WF rats transplanted with nonencapsulated islets ($n=6$), and immunized rats transplanted with nonencapsulated islets ($n=6$) are compared. Representative images from ELISpot wells are shown under each figure. *$p<0.05$.

In our experimental study, five of six immunized recipients maintained alloreactive antibodies for the 6-month study period. The TheraCyte™ device excludes immune cells, but the pore size indicates that the membrane could be permeable to antibodies. Thus, in immunized recipients, donor-specific antibodies could theoretically enter the device and cause graft damage by complement fixation. In addition, the activation of the complement pathways could attract inflammatory cells to the surrounding tissue, which would further harm the graft. High levels of inflammatory cytokines may also upregulate antigen expression, for example, MHC classes I and II on islets (6), rendering the graft more susceptible to antibody-mediated damage. However, our histological evaluation

revealed no significant inflammation around the devices in naive and in immunized recipients. Additionally, we found no signs of antibody-mediated damage to the encapsulated islets as indicated by the lack of difference of the absolute volume of total tissue and the fractional volume of endocrine and fibrotic tissues between immunized and nonimmunized recipients. The absence of tissue damage inside the devices may be explained by the slow passage IgG antibodies (1). Furthermore, the use of a preimplantation technique, where devices are implanted 3 months before islet transplantation, allows the transplantation of islets into a low inflammatory environment.

After immunization, in vitro $CD8^+$ T-cell activity against donor splenocytes was significantly increased. To evaluate whether immunization was sustained throughout the study period, the IFN-γ response by alloreactive $CD8^+$ T-cells was evaluated at the time of sacrifice. Significant differences were noted between immunized and nonimmunized hosts receiving encapsulated islets. Thus, our data indicated that the TheraCyte™ device could protect the islet allograft for an extended period of time in immunized hosts.

Nonimmunized recipients that were transplanted with islets in the device were protected from sensitization as previously described (19). Whether the encapsulated islets can maintain or enhance an immune response in immunized recipients should be examined further. We found that the levels of antidonor antibodies in immunized recipients decreased over time. On the other hand, memory $CD8^+$ T-cell activity was maintained but our study did not suggest that encapsulated islets enhanced T-cell activity over time. Cells from immunized rats that received nonencapsulated islets under the kidney capsule produced the highest levels of IFN-γ.

The metabolic control provided by the encapsulated islet grafts was similar in naive and immunized recipients. However, during OGTT, rats transplanted with encapsulated islets showed higher variations in blood glucose and plasma insulin levels compared to healthy controls. A recent study demonstrated that transplanted islets have a reduced function compared to endogenous islets in the pancreas (4). This reduction may be caused by an altered structure of transplanted islets. Our findings may also reflect variations in both the quality of the islet grafts and in the neovascularization around the device. In addition, the subcutaneous implantation of the TheraCyte™ device may result in a slower insulin response to changes in blood glucose level. Thus, our findings indicate that the metabolic control induced by encapsulated islets may be different from that observed in healthy controls. Several factors may contribute to these differences in function. More extensive studies on the metabolic function of islets implanted subcutaneously into TheraCyte™ devices are currently ongoing.

Histological examination after 6 months revealed significant fibrosis inside the devices. Still, blood sugar levels were maintained at normal levels. The development of fibrosis inside the device was not related to the immunization status of the rats. We have also observed similar findings in previous studies that included the transplantation of encapsulated islets into nude mice (7,19). This finding is concerning in that it may pose a threat to long-term graft function. It may be hypothesized that the fibroblasts of donor origin expand within the empty spaces within devices. This is an important question to be addressed before these devices are useful in the clinic.

In conclusion, this study shows that the TheraCyte™ device protected encapsulated islet grafts in immunized recipients despite the maintained presence of alloantibodies and alloreactive $CD8^+$ T-cells. Considering that many type 1 diabetic patients who would benefit from an islet transplant are already immunized, these results lend further support for macroencapsulation as a promising strategy to be used clinically.

ACKNOWLEDGMENTS: Financial support was provided through the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and the Karolinska Institutet (project numbers 20060710, 20080443), the Swedish Research Council (52120083389), research grants from the Karolinska Institutet, the Strategic Research Programme in Diabetes at Karolinska Institutet, the Foundation for International Surgical Cooperation, the Foundation for Oncological Cooperation, The Swedish Child Diabetes Foundation. The TheraCyte™ devices used in this study were kindly provided by TheraCyte Inc., Laguna Hills, CA, USA. The authors declare no conflict of interest.

REFERENCES

1. Brauker, J. H.; Carr-Brendel, V. E.; Martinson, L. A.; Crudele, J.; Johnston, W. D.; Johnson, R. C. Neovascularization of synthetic membranes directed by membrane microarchitecture. J. Biomed. Mater. Res. 29:1517–1524; 1995.
2. Brauker, J. H.; Martinson, L. A.; Young, S. K.; Johnson, R. C. Local inflammatory response around diffusion chambers containing xenografts. Nonspecific destruction of tissues and decreased local vascularization. Transplantation 61:1671–1677; 1996.
3. Campbell, P. M.; Senior, P. A.; Salam, A.; Labranche, K.; Bigam, D. L.; Kneteman, N. M.; Imes, S.; Halpin, A.; Ryan, E. A.; Shapiro, A. M. J. High risk of sensitization after failed islet transplantation. Am. J. Transplant. 7:2311–2317; 2007.
4. Cornolti, R.; Cattaneo, I.; Trudu, M.; Figliuzzi, M.; Remuzzi, A. Effect of islet transplantation on metabolic glucose control in rats with diabetes. Diabetes Technol. Ther. 11:805–811; 2009.
5. Harlan, D. M.; Kenyon, N. S.; Korsgren, O.; Roep, B. O. Current advances and travails in islet transplantation. Diabetes 58:2175–2184; 2009.
6. Jackson, A. M.; Connolly, J. E.; Matsumoto, S.; Noguchi, H.; Onaca, N.; Levy, M. F.; Naziruddin, B. Evidence for induced expression of HLA class II on human islets: Possible mechanism for HLA sensitization in transplant recipients. Transplantation 87:500–506; 2009.

7. Jia, X.; Sharma, A.; Kumagai-Braesch, M.; Wernerson, A. M.; Sörenby, A. K.; Yamamoto, S.; Wang, F.; Tibell, A. B. Exendin-4 increases the expression of hypoxia-inducible factor-1α in rat islets and preserves the endocrine-cell volume of both free an macroencapsulated islet grafts. Cell Transplant. 21(6):1269–1283; 2012.
8. Jones, K. S.; Sefton, M. V.; Gorczynski, R. M. In vivo recognition by the host adaptive immune system of microencapsulated xenogeneic cells. Transplantation 78:1454–1462; 2004.
9. Kessler, L.; Parissiadis, A.; Bayle, F.; Moreau, F.; Pinget, M.; Froelich, N.; Cazenave, J. P.; Berney, T.; Benhamou, P. Y.; Hanau, D. Evidence for humoral rejection of a pancreatic islet graft and rescue with rituximab and iv immunoglobulin therapy. Am. J. Transplant. 9:1961–1966; 2009.
10. Loudovaris, T.; Charlton, B.; Hodgson, R. J.; Mandel, T. E. Destruction of xenografts but not allografts within cell impermeable membranes. Transplant. Proc. 24:2291–2292; 1992.
11. Loudovaris, T.; Mandel, T. E.; Charlton, B. $CD4^+$ T-cell mediated destruction of xenografts within cell-impermeable membranes in the absence of $CD8^+$ T-cells and B-cells. Transplantation 61:1678–1684; 1996.
12. McKenzie, A. W.; Georgiou, H. M.; Zhan, Y.; Brady, J. L.; Lew, A. M. Protection of xenografts by a combination of immunoisolation and a single dose of anti-CD4 antibody. Cell Transplant. 10:183–193; 2001.
13. Merville, P.; Pouteil-Noble, C.; Wijdenes, J.; Potaux, L.; Touraine, J. L.; Banchereau, J. Detection of single cells secreting IFN-γ, IL-6, and IL-10 in irreversibly rejected human kidney allografts, and their modulation by IL-2 and IL-4. Transplantation 55:639–646; 1993.
14. Pathan, A. A.; Wilkinson, K. A.; Wilkinson, R. J.; Latif, M.; McShane, H.; Pasvol, G.; Hill, A. V.; Lalvani, A. High frequencies of circulating IFN-γ-secreting CD8 cytotoxic T-cells specific for a novel MHC class I-restricted mycobacterium tuberculosis epitope in m. Tuberculosis-infected subjects without disease. Eur. J. Immunol. 30:2713–2721; 2000.
15. Rafael, E.; Wu, G. S.; Hultenby, K.; Tibell, A.; Wernerson, A. Improved survival of macroencapsulated islets of Langerhans by preimplantation of the immunoisolating device: A morphometric study. Cell Transplant. 12:407–412; 2003.
16. Ryan, E. A.; Paty, B. W.; Senior, P. A.; Bigam, D.; Alfadhli, E.; Kneteman, N. M.; Lakey, J. R.; Shapiro, A. M. Five-year follow-up after clinical islet transplantation. Diabetes 54:2060–2069; 2005.
17. Safley, S. A.; Kapp, J. A.; Weber, C. J. Proliferative and cytokine responses in CTLA4-Ig-treated diabetic NOD mice transplanted with microencapsulated neonatal porcine ICCs. Cell Transplant. 11:695–705; 2002.
18. Sharma, A.; Sorenby, A.; Wernerson, A.; Efendic, S.; Kumagai-Braesch, M.; Tibell, A. Exendin-4 treatment improves metabolic control after rat islet transplantation to athymic mice with streptozotocin-induced diabetes. Diabetologia 49:1247–1253; 2006.
19. Sorenby, A. K.; Kumagai-Braesch, M.; Sharma, A.; Hultenby, K. R.; Wernerson, A. M.; Tibell, A. B. Preimplantation of an immunoprotective device can lower the curative dose of islets to that of free islet transplantation: Studies in a rodent model. Transplantation 86:364–366; 2008.
20. Sorenby, A. K.; Wu, G. S.; Zhu, S.; Wernerson, A. M.; Sumitran-Holgersson, S.; Tibell, A. B. Macroencapsulation protects against sensitization after allogeneic islet transplantation in rats. Transplantation 82:393–397; 2006.
21. Sweet, I. R.; Yanay, O.; Waldron, L.; Gilbert, M.; Fuller, J. M.; Tupling, T.; Lernmark, A.; Osborne, W. R. Treatment of diabetic rats with encapsulated islets. J. Cell. Mol. Med. 12:2644–2650; 2008.
22. Tibell, A.; Rafael, E.; Wennberg, L.; Nordenstrom, J.; Bergstrom, M.; Geller, R. L.; Loudovaris, T.; Johnson, R. C.; Brauker, J. H.; Neuenfeldt, S.; Wernerson, A. Survival of macroencapsulated allogeneic parathyroid tissue one year after transplantation in nonimmunosuppressed humans. Cell Transplant. 10:591–599; 2001.
23. Toso, C.; McCall, M.; Emamaullee, J.; Merani, S.; Davis, J.; Edgar, R.; Pawlick, R.; Kin, T .; Knudsen, L. B.; Shapiro, A. M. Liraglutide, a long-acting human glucagon-like peptide 1 analogue, improves human islet survival in culture. Transpl. Int. 23:259–265; 2010.
24. Weibel, E. R. Morphometry of the human lung: The state of the art after two decades. Bull. Eur. Physiopathol. Respir. 15:999–1013; 1979.
25. Yang, Z.; Chen, M.; Fialkow, L. B.; Ellett, J. D.; Wu, R.; Nadler, J. L. Survival of pancreatic islet xenografts in NOD mice with the theracyte device. Transplant. Proc. 34:3349–3350; 2002.