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Zhou, D., A. M. Sun, X. Li, S. N. Mamujee, I. Vacek, J. Georgiou, and M. B. Wheeler. In vitro and in vivo evaluation of insulin-producing βTC6-F7 cells in microcapsules. *Am. J. Physiol.* 274 (*Cell Physiol.* 43): C1356–C1362, 1998.—In the present study, the insulin secretory capacity of βTC6-F7 cells in microcapsules was evaluated. The cell mass within capsules was found to expand in a three-dimensional fashion, in contrast to cells seeded on plates that grew as a monolayer. In in vitro studies, both free and encapsulated cells were found to secrete insulin in the absence of glucose, at 13.6 ± 1.1 and 14.5 ± 0.9 ng·10^6 cells^{-1}·60 min^{-1}, respectively, with the response rising to a maximum of 26.0 ± 0.8 and 31 ± 2.3 ng·10^6 cells^{-1}·60 min^{-1} in the presence of 16.8 mM glucose. Encapsulated cells were able to produce Ca^{2+} responses in the presence of KCl (50 mM) and BAY K 8644 (100 μM). In in vivo studies, intraperitoneal transplantation of 3.0 ×10^6 microencapsulated cells into mice (n = 5) with streptozotocin-induced diabetes resulted in the restoration of normoglycemia up to 57 days. Insulin concentrations rose from 0.4 ± 0.1 ng/ml before the graft administration to 2.2 ± 0.8 ng/ml after the transplantation in the normoglycemic recipients. An oral glucose challenge in transplant recipients demonstrated a flat glucose response, suggesting extremely high glucose clearance rates. These data demonstrate the potential use of the immunoisolated β-cell lines for the treatment of diabetes.

insulin-producing cells; microencapsulation; xenografts; transplantation

THE RESULTS OF THE recent Diabetes Control and Complications Trial (3) demonstrate that there is a major impact of poor metabolic control on the rate of appearance of diabetic complications and that hyperglycemia is the key factor in the initiation of various pathological processes. Therefore, the tight control and maintenance of euglycemia should be the fundamental measure for preventing or delaying the development of diabetic complications. Consequently, it has become imperative to develop methods, applicable early in the course of the disease, for obtaining perfect metabolic control without increasing the risk of severe hypoglycemia. Currently, the transplantation of islet tissue, either as a whole pancreas or as isolated islets, has been pursued because this technique can provide normal blood glucose control and thus have the potential to prevent or reduce diabetic complications (3, 19, 22).

To overcome the problem of immunorejection and the need for immunosuppression and to circumvent the potential for disease recurrence, the concept of immunoisolation has been advanced. In our approach to immunoisolation, we have developed alginate-polylysine-alginate biocompatible capsules to enclose individual pancreatic islets (5, 9, 12, 13, 16). In these earlier studies, both allografts and xenografts of microencapsulated isolated pancreatic islets were shown to reverse diabetes in long-term experiments in spontaneously diabetic as well as chemically induced diabetic rodents (5, 9, 12, 13, 16). These studies demonstrate the potential for the use of allo- and xenotransplantation as therapeutic alternatives to exogenous insulin therapy in insulin-dependent diabetics.

Although there is little question that transplanted islets offer a potential alternative to exogenous insulin therapy, large-scale islet isolation and cryopreservation, however, are both technically difficult and expensive. In addition, consistent and reliable performance of islet allografts and xenografts, from batch to batch, may be difficult to achieve. Insulin-secreting cell lines derived from β-cells represent a potential alternative approach to pancreatic islet transplantation. These cells can be grown inexpensively and in unlimited quantity. Several glucose-responsive β-cell lines have been produced through the culturing of islet tumors; however, these cell lines are, in general, phenotypically unstable as evidenced by a shift in glucose responsivity and/or diminished insulin output through passage (4, 7, 17, 20, 21). Recently, Knaack et al. (8) reported the cloning of a phenotypically stable (>55 passages) β-cell line, βTC6-F7. This cell line has been shown to have remarkably similar characteristics to β-cells of pancreatic islets, including appropriate glucose-induced insulin responsivity (5–30 mmol range) and the expression of GLUT-2 but not GLUT-1 glucose transporters, with high-glucokinase and low-hexokinase activities, respectively. In the present study, we evaluated the βTC6-F7 cells both in vitro and in vivo and tested the hypothesis that βTC6-F7 cells microencapsulated and transplanted into diabetic mice would control hyperglycemia in long-term experiments. The results of these studies are described.

MATERIALS AND METHODS

Cell culture. βTC6-F7 cells were grown and passaged as previously described (8) with the following modifications: cells were grown in RPMI 1640 medium (GIBCO) containing 5.5 mM glucose and supplemented with 10% FCS and 2 mM L-glutamine.

Cell encapsulation. The encapsulation technique was a modification of the method of Lim and Sun (11). This modification involved the use of an electrostatic droplet generator (6), which produces smaller, stronger, and more uniform capsules compared with the older air-jet technique. The cells were suspended in 1.5% (wt/vol) purified sodium alginate (Kelco Gel LV, Kelco, San Diego, CA) at a concentration of ~1.5 × 10^7 cells/ml. Spherical droplets were formed by the electrostatic field interaction coupled with syringe pump extrusion and

were collected in a 100 mM calcium lactate solution. The gelled droplets were suspended in 0.05% polylysine (Sigma, molecular mass = 22–24 kDa) for 5 min. The droplets were washed with 0.9% saline and suspended in 0.15% sodium alginate for 5 min. After wash with 0.9% saline, the capsules were allowed to react with 55 mM sodium citrate for 5 min and finally washed with 0.9% saline and with culture medium. On average, capsules were designed to contain ~300 cells and have an average diameter of 0.25–0.35 mm.

Cell viability assays. To examine cell viability at different stages of culture (2 through 6 wk) cells were imaged by loading 1 μM calcein-AM (Molecular Probes, Eugene, OR) with a final concentration of 0.1% pluronic acid. A confocal laser scanning microscope (Bio-Rad 600) was used to analyze viable cells using the 488-nm line of the argon laser, and emitted fluorescence was detected through a low-pass filter with cutoff at 515 nm. To examine nonviable cells, encapsulated βTC6-F7 cells were stained with 5 μM propidium iodide (Sigma) for 10 min. Propidium iodide was excited using the 514-nm laser line, and the emitted fluorescence was detected through a 550-nm long-pass filter.

In vitro insulin secretion studies. For initial characterization of βTC6-F7 cells, insulin secretion experiments were performed. Briefly, cells at *passages 40–42* were plated in 24-well plates at a density of 100,000 cells per well and cultured for an additional 48 h. Cells were then preincubated in zero glucose RPMI 1640 (0.1% BSA, 0.05% bacitracin) for 60 min, followed by a 60-min stimulation with various concentrations of glucose (0–16.8 mM, 2 ml total volume). With encapsulated cells, groups of 40 microcapsules, each initially containing ~300 cells, were cultured in tissue culture multiwell plates for 48 h following encapsulation. After a 60-min preincubation period in RPMI 1640 (0 glucose), the encapsulated cells were exposed to glucose (2 ml total volume) for 60 min. Samples of the medium were collected following glucose exposure and stored at −2°C before assay. In each case, the insulin content of the samples was determined by radioimmunoassay (rat insulin kit, Linco Research, St. Louis, MO); results (in ng/ml) were expressed as means ± SE of at least three independent experiments.

Free cytosolic Ca^{2+} measurements. A confocal laser scanning microscope (Bio-Rad 600) was used to analyze Ca^{2+} fluxes in encapsulated βTC6-F7 cells after ~2 wk under standard culture conditions, as described. Cells were loaded with 10 μM fluo 3-AM (Molecular Probes) for 1 h in DMEM, 0.5% DMSO, and 0.1% pluronic acid. Subsequently, cells were washed twice in DMEM and changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were analyzed in the presence of 50 mM KCl or BAY K 8644 (100 μM) for 250 ms. Fluo 3 was excited using the 488-nm line of the argon laser, and emitted fluorescence was detected through a low-pass filter with cutoff at 515 nm. The detected signals were digitized, forming an eight-bit number (0–255) to represent each pixel. Images were thus collected digitally, and a false color scale was generated for quantitative purposes, where blue corresponds to lower and red to higher Ca^{2+} levels. The changes in fluorescence were measured using CFOCAL, a program for PC analysis and the preparation of confocal images (written by T. A. Goldthorpe, Department of Physiology, University of Toronto). Changes in Ca^{2+} fluorescence (F) are expressed as $\Delta F/F = (F - F_{rest})/F_{rest}$, where F_{rest} is the resting fluorescence level.

Transplantation studies. For the induction of diabetes, animals (C57/BL mice; Charles River, St. Constant, PQ, Canada) were administered streptozotocin intravenously at 185 mg/kg; subsequently, these animals were considered diabetic and therefore suitable for transplantation after registering three consecutive, nonfasting blood glucose measurements above 20 mM. Before the transplant, the microencapsulated cells were cultured overnight under standard culture conditions. With the use of light anesthesia, groups of mice with streptozotocin-induced diabetes received a single transplant of either 3.0×10^6 ($n = 5$), 1.5×10^6 ($n = 3$), or 0.75×10^6 ($n = 2$) microencapsulated cells, ~300 cells per capsule, *passages 40–42*. A control group of diabetic mice ($n = 3$) received corresponding numbers of free, unencapsulated cells. Another diabetic control group ($n = 3$) received equal numbers of empty capsules. The grafts were administered by intraperitoneal injection using an 18-gauge cannula. At regular intervals (2–3 days), blood samples were taken via tail vein from all animals for blood glucose monitoring and analyzed with a glucometer (Miles, Toronto, ON, Canada). To confirm that the normoglycemic condition of these diabetic animals indeed resulted from the graft of the microencapsulated cells, capsules were removed from the peritoneal cavities of two normoglycemic, randomly selected recipients of 3.0×10^6 microencapsulated cells 35 days after transplantation. The procedure was performed under ether anesthesia. The microcapsules were retrieved by repeated washes with saline inside the peritoneal cavity.

Glucose tolerance test. Oral glucose tolerance tests (OGTTs) were administered to transplant recipients approximately 1 wk after normoglycemia had been established as a result of the grafts. Nondiabetic animals were used as controls. Before the OGTTs, all experimental animals were fasted overnight. In the OGTT, 1.5 mg of glucose per gram of body weight were instilled orally into each experimental mouse, and blood glucose concentrations were established over a 120-min period.

Statistical analysis. Results are given as means ± SE of at least three independent observations unless otherwise stated. Data were analyzed with the Student's *t*-test and χ^2 test for statistical significance. $P < 0.05$ was considered significant.

RESULTS

Imaging of encapsulated βTC6-F7 cells. Encapsulated cells cultured for 2–6 wk were examined by confocal microscopy to observe their growth activity in microcapsules (Fig. 1). Cells initially formed several small aggregates within 1–2 wk (Fig. 1*Ai*) that would interfuse primarily into one to a few large-core aggregates within 3–4 wk (Fig. 1, *Bi*, *Ci*, and *Ei*). After 4 wk in culture, the majority of capsules contained primarily one central aggregate, and, by 5–6 wk, the aggregate had expanded to fill the capsule (Fig. 1, *Di*, *Fi*, and *Gi*). We used three approaches to determine encapsulated βTC6-F7 viability within the capsule. First, live cells were visualized by calcein fluorescence (Fig. 1, *Aii–Dii*). This method was employed because a laser confocal microscope can be used to visualize the viability of cells within the central core of the capsule, on the basis of the premise that enzymatic activity is required to activate fluorescence of calcein-AM and cell membrane integrity is required for its retention (14, 15) (Fig. 1, *Aii–Dii*). As shown, the majority of cells within the capsules were labeled (viable cells emitting a green fluorescence); however, there was a tendency for cells in the central core of the clusters to not label as strongly. A trypan blue exclusion test was also performed on encapsulated cells after 3 and 6 wk in culture. This test revealed that at 3 wk of culture more than 90% of cells

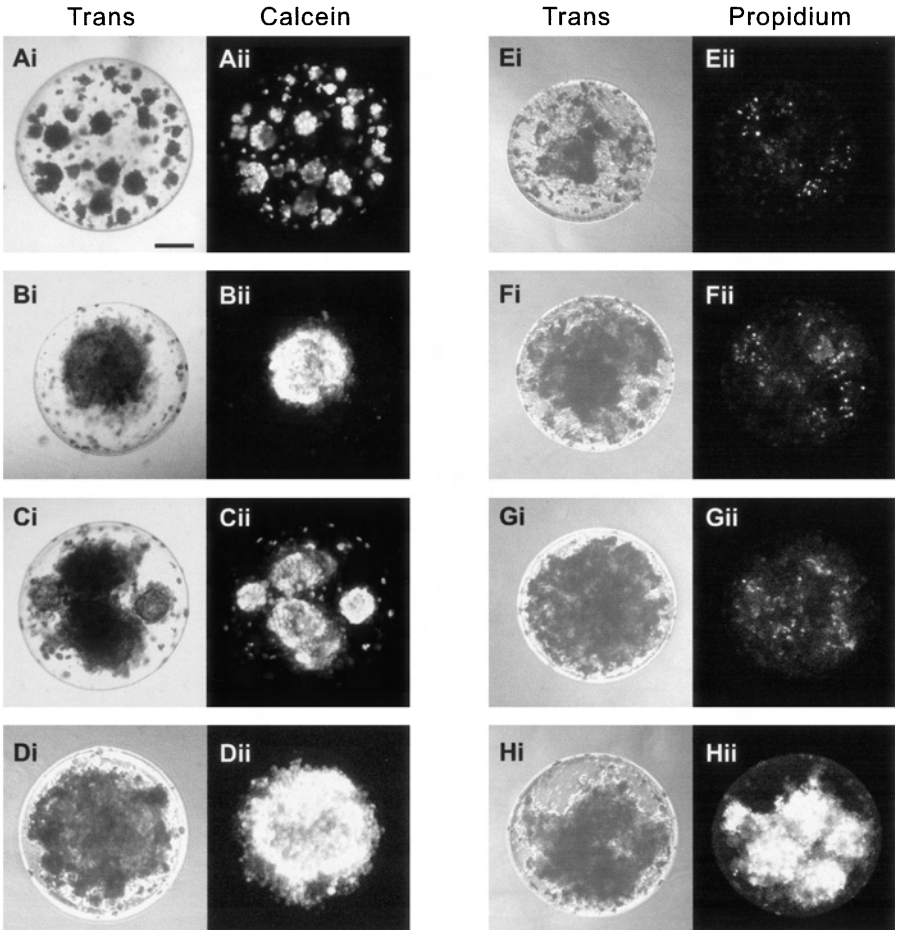

Fig. 1. Photomicrographs and fluorescent images of microencapsulated βTC6-F7 cells. Cells were encapsulated and cultured for 2–6 wk to observe their growth activity in microcapsules. Scale bar = 100 μm. Trans images (*i*) refer to a light images acquired using a transillumination adapter. Cells initially formed several small aggregates within 2 wk (*Ai*) that would form primarily into a few large-core aggregates within 4 wk (*Bi*, *Ci*, *Ei*). By 6 wk in culture, the majority of capsules contained primarily 1 central core aggregate that would ultimately fill the capsule (*Di*, *Fi–Hi*). Simultaneous to trans micrographs, images of calcein fluorescence from the capsule series are shown (*Aii–Dii*). Live cells are detected by fluorescence using a confocal microscope. Similarly, propidium iodide fluorescence images are shown (*far right*) for a second series of capsules (*Eii–Hii*). Cells with labeled nuclei are not viable. *Hi* and *Hii* are representative of a capsule series cultured at room temperature in serum-free media for 2 days before the experiment. Note the intense nuclear fluorescence (*Hii*), indicating poor viability.

were viable and at 6 wk ~80% of cells appeared viable, in agreement with the calcein results. To assess viability in an alternative way, expired or defunct cells were visualized using propidium iodide. This red fluorescent DNA binding agent is cell impermeant and thus will only gain access to cells with compromised membranes (1). As shown in Fig. 1, *Eii–Gii*, a small proportion of cells have labeled nuclei, and there was no correlation between labeling and cells within the central core of the cell clusters. This pattern of fluorescence is in contrast to cells cultured at room temperature in serum-free media for 2 days, in which most of the cells (Fig. 1*Hii*) have labeled nuclei. These studies demonstrate that the majority of cells up to 6 wk postencapsulation are viable, which is quite surprising given that the cells do poorly in monolayer culture when confluence approaches values >70% (unpublished results).

In vitro glucose challenge experiments. The results of the in vitro glucose challenge study comparing microencapsulated and free cells are summarized in Fig. 2. In the absence of glucose, the encapsulated cells and the free cells secreted 14.9 ± 0.9 and 13.6 ± 1.1 ng insulin·10^6 cells^{-1}·60 min^{-1}, respectively. The exposure of the cells to 2.8 mM glucose resulted in insulin secretion of 19.4 ± 2.6 and 17 ± 0.62 ng·10^6 cells^{-1}·60 min^{-1} for encapsulated and free cells, respectively. On exposure to 5.6 mM glucose, the encapsulated and free cells secreted insulin at a rate of 21.1 ± 2.5 and 22.7 ± 0.49 ng·10^6 cells^{-1}·60 min^{-1}, respectively. Finally, at the high-glucose concentration of 16.8 mM, the insulin secretion for encapsulated and free cells amounted to 31.0 ± 2.3 and 26.0 ± 0.8 ng·10^6 cells^{-1}·60 min^{-1}, respectively. These results demonstrate that free and encapsulated cells respond similarly to a glucose stimulus.

Measurement of $[Ca^{2+}]_i$. Cells in capsules were examined in vitro for their ability to depolarize and regulate $[Ca^{2+}]_i$ in response to test agents (Figs. 3 and 4 and Table 1). In cells loaded with fluo 3-AM, the majority was observed to have similar resting Ca^{2+} fluorescence intensities; however, there was a trend toward cells in the interior of larger aggregates to have significantly lower resting fluorescence intensities (Fig. 3*B*, summarized in Table 1). We attribute this lower resting intensity to the reduced efficiency with which fluo 3-AM penetrates the centers of the these large aggregates and not to a reduced viability of the central portions of the cores, based on the studies with calcein and propidium iodide. In response to 50 mM KCl (Fig. 3*C*), delivered by micropipette (Figs. 3 and 4*A*) to the interior of a capsule, the majority of cells showed an increase in Ca^{2+} fluorescence intensity, as depicted using a false color scale. Fluorescence intensities were found to return to prestimulatory levels within minutes of the KCl stimulus (data not shown). The changes in fluorescence for four isolated cells (2 from central and 2

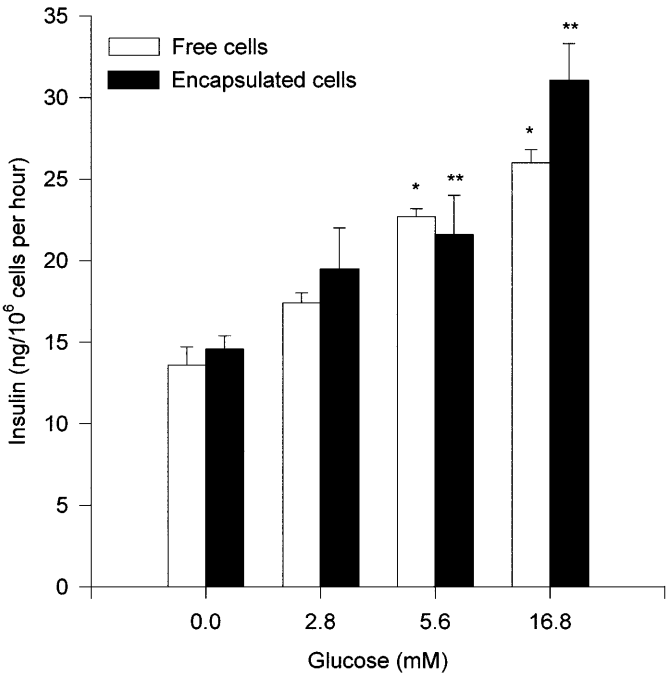

Fig. 2. In vitro glucose challenge study comparing free and encapsulated cells. Free or encapsulated cells were preincubated in 0 glucose RPMI 1640 (0.1% BSA, 0.05% bacitracin) for 60 min, followed by a 60-min stimulation with various concentrations of glucose (0–16.8 mM). With free cells, $*P < 0.05$ for insulin secretion at 5.6 and 16.8 mM vs. insulin secretion at 0 mM glucose. $**P < 0.05$ for encapsulated cell insulin secretion at 5.6 and 16.8 mM glucose vs. secretion at 0 mM glucose ($n = 5$ independent observations per group).

from the periphery of large aggregates) were normalized to respective control values (ΔF/F) and plotted against time (Fig. 4*A*). The pattern of fluorescence, typical of a depolarization event, was monophasic, with a rapid transient peak of Ca^{2+}. The net increase above basal levels was similar for cells in the core or periphery of large aggregates (Table 1). In Fig. 4*B*, KCl delivered to the medium bathing capsules gave a similar response from representative responders, but the response was slightly delayed. The L-type Ca^{2+} channel agonist BAY K 8644 (100 μM) delivered to the bathing medium also elicited Ca^{2+} responses (Fig. 4*C*). These studies suggest that most of the cells express functional voltage-dependent Ca^{2+} channels, as well as the appropriate intracellular mechanisms for removing Ca^{2+} from the cytoplasm.

In vivo studies with transplanted capsules. The intraperitoneal transplantation of microencapsulated cells with the highest dose of 3×10^6 cells resulted in the restoration of normoglycemia in all experimental animals ($n = 5$) within 5–7 posttransplantation days (Table 2). The blood glucose profiles of these animals are shown in Fig. 5. One out of three mice that received 1.5×10^6 microencapsulated cells became normoglycemic (with blood glucose concentrations of <6.0 mM), whereas only a partial lowering of blood glucose levels (6–11 mM) was observed in the other two recipients. Finally, only a partial lowering of the blood glucose concentrations was observed in the two recipients with the lowest dose, 0.75×10^6 microencapsulated cells. In the three recipients with 3.0×10^6 free unencapsulated βTC6-F7 cells (i.e., an equivalent of the highest dose of encapsulated cells), normoglycemia was restored within 6 posttransplantation days; however, during the next 7 days, the blood glucose concentrations returned to the original hyperglycemic range, thus indicating the destruction of the graft by the immune system of the recipient. No change in the diabetic hyperglycemia was observed in the recipients of empty capsules. The duration of normoglycemia in the five recipients of the highest dose of the microencapsulated cells was at least 35 days (on *day 35*, 2 animals of this group had the capsules surgically removed), with the three remaining normoglycemic from 55 to 57 days, at which time the grafts failed (Fig. 5). The removal of the microcapsules at 35 days posttransplantation resulted in a return to hyperglycemia within 24 h. The recovered capsules were free of cell overgrowth and physically intact, with enclosed clusters of cells clearly visible. The body weights of the transplant recipients in which normoglycemia had been established increased during the period of normoglycemia, whereas hyperglycemic animals lost weight. Although the serum insulin concentrations in the diabetic mice before the transplants averaged 0.4 ± 0.1 ng/ml following the graft administration, the insu-

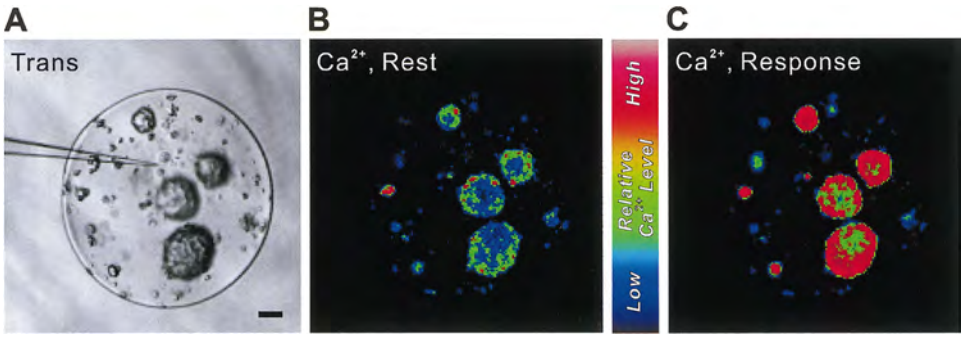

Fig. 3. Ca^{2+} responses of encapsulated βTC6-F7 cells. *A*: nonconfocal image acquired using the transmitted light attachment of the Bio-Rad 600 microscope, revealing a capsule containing βTC6-F7 cells (~2 wk postencapsulation). Note the microelectrode that has been inserted into the capsule. Scale bar = 50 μm. *B*: confocal image of the same encapsulated cells loaded with fluo 3-AM, showing resting Ca^{2+} levels. Relative Ca^{2+} fluorescence appears in false color (see bar at *right*). *C*: application of KCl (50 mM) induced a Ca^{2+} response in most of the cells. Data are representative of 5 capsule experiments.

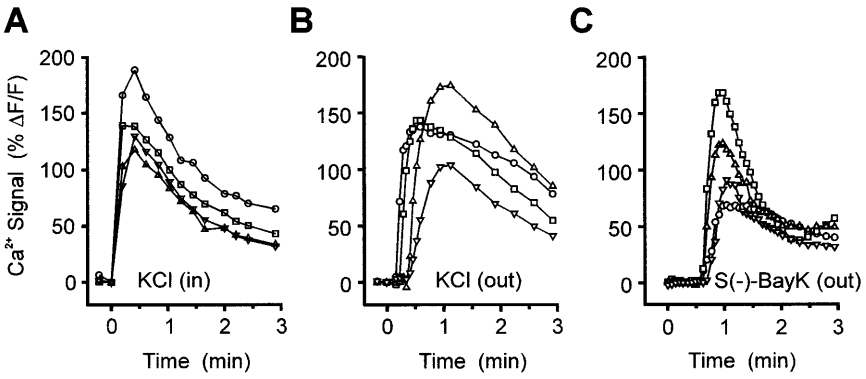

Fig. 4. Time course of Ca^{2+} responses of capsules containing βTC6-F7 cells (measure in ΔF/F, where F is the detected fluorescence). *A*: time course of Ca^{2+} signals evoked by KCl application (50 mM) to the interior of the capsules. Each symbol in *A–C* represents a region of analysis with 2 cells from the central cores (triangles) and 2 from the periphery of large aggregates (squares and circles). Changes in Ca^{2+} from 4 regions were analyzed and plotted with respect to time, with KCl being applied at time = 0 min. *B*: time course experiment with KCl being applied to the bathing solution. Independent areas within 4 capsules were analyzed. *C*: Ca^{2+} responses from 4 capsules on addition of the L-type Ca^{2+} channel agonist BAY K 8644 (100 μM). Agonist was released at time = 0 min.

lin concentration increased to 2.2 ± 0.8 ng/ml ($n = 5$, $P < 0.05$ compared with the pretransplant concentration) in the recipients in which normoglycemia was achieved. The average serum insulin concentration in healthy control animals was determined at 2.0 ± 0.3 ng/ml. The OGTTs indicated extremely high glucose clearance rates in the animals in which normoglycemia had been restored as a result of the graft administration (Fig. 6). These clearance rates were reminiscent of glucose clearance rates of human patients suffering from insulinoma.

DISCUSSION

Clinical transplantation of human islets from cadavers proved that this can normalize hyperglycemia in diabetic patients (23). However, the limited supply of human islets, especially when the procurement of cadaver pancreata is not controlled, makes their use impractical. Consequently, if the mass clinical transplantation of pancreatic islets into human diabetics is to become a reality, xenografts of animal islets will have to be used. We have shown in our past experiments that xenotransplants do work in animal models (5, 9, 12, 13, 16). Thus clinical transplantation of animal pancreatic endocrine tissue may be used in the near future as a novel treatment for diabetes. Although this approach could revolutionize the management of diabetes mellitus, the use of animal tissue would also present a number of challenges. The possible existence of animal pathogens transmissible to the human graft recipients must be carefully considered. The technique of islet isolation from the pig, which now seems to represent the most likely candidate for donor tissue, involves a number of technical difficulties. The process of isolation and purification of porcine islets is expensive and labor intensive. Although the use of animal islets may yet prove to be the right concept for the treatment of diabetes, it also seems appropriate to search for possible alternatives. Because our concept of immunoisolation by microencapsulation of cells and tissues has a vast scope of possible applications, the use of bioengineered insulin-producing cells becomes very attractive.

As a model, we have selected an insulin-producing clonal line βTC6-F7, originally derived from insulinomas arising in transgenic mice expressing the SV40 T antigen gene under control of the insulin promoter. Because βTC6-F7 cells are derived from the B6D2F1/J mouse strain and C57/BL mice were used as transplant recipients, allotransplantation was performed in this study. After microencapsulation, the βTC6-F7 cell mass expanded within the capsules, displaying a pattern of distribution that was substantially different from that of the free, unencapsulated cells (Fig. 1). In monolayer culture, cells would grow to ~70% of confluence at which point the cells would quite rapidly start necrotizing. The encapsulated cells grew in a three-dimensional fashion, initially in a few aggregates that would later (at 4–6 wk) expand and fill the entire capsule, visualized under light microscopy as a solid mass. Confocal microscopy in combination with the appropriate fluorescent dyes was used to test for viability and to demonstrate that the cells, including those at the most central point within the capsule, were viable. These qualitative data are supported by trypan blue exclusion experiments that show that the majority of the cells (>80%) were still living at 6 wk of culture.

Table 1. *Comparison of resting and peak $[Ca^{2+}]_i$ responses to KCl from βTC6-F7 cells located on the periphery or at the core of large cell aggregates*

	Resting $[Ca^{2+}]_i$, pixel intensity	Peak $[Ca^{2+}]_i$, pixel intensity	Ca^{2+} Signal, %ΔF/F
Peripheral cells	95 ± 8	206 ± 10	117 ± 7
Central cells	65 ± 9*	151 ± 17†	133 ± 8

Values are means ± SE, obtained as described in MATERIALS AND METHODS; $n = 3$ for each group. *$P < 0.01$ central vs. peripheral cells at resting and †$P < 0.05$ for central vs. peripheral at peak response. Results for %ΔF/F (where ΔF/F is the change in fluorescence divided by the measured fluorescence) not considered significant, $P > 0.225$.

Table 2. *Restoration of normoglycemia within 5–7 days in streptozotocin-induced diabetic mice transplanted with microencapsulated βTC6-F7 cells*

Group	Number of Cells	Normoglycemic Recipients	Partially Normoglycemic	Total
1	3.0×10^6	5	0	5*
2	1.5×10^6	1	2	3
3	0.75×10^6	0	2	2
4	0	0	0	3

*$P < 0.001$ as compared with *group 4*.

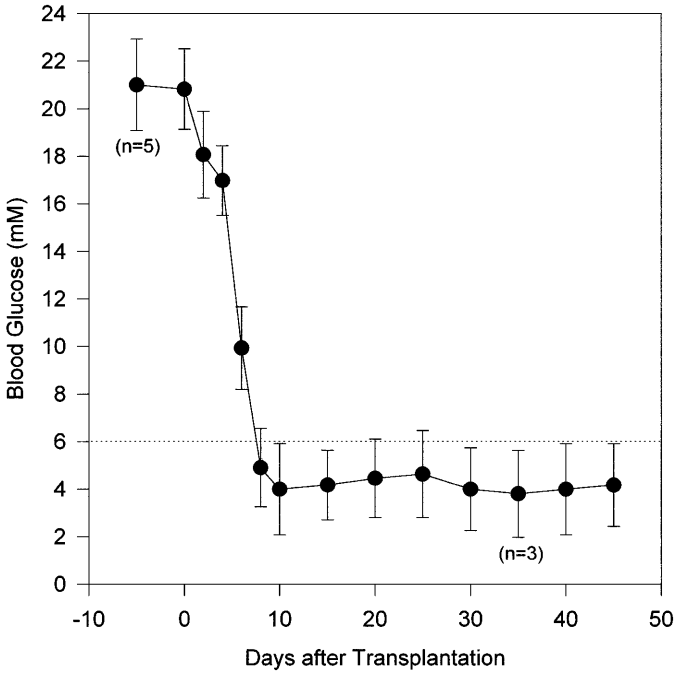

Fig. 5. Blood glucose profiles of mice transplanted with microencapsulated βTC6-F7 cells. Streptozotocin-induced diabetic mice (initially, $n = 5$) received a single transplant of 3.0×10^6 microencapsulated cells, ~300 cells per capsule, administered by intraperitoneal injection. Capsules were removed from 2 animals on *day 35*. With the remaining animals, implants failed to normalize glucose on *days 50*, *55*, and *57*. Data are presented as means ± SE.

In response to KCl and several secretagogues that stimulate β-cells, the cell depolarizes, followed by an influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (reviewed in Ref. 18). The increase in free $[Ca^{2+}]_i$ is thought to be a trigger for insulin secretion (18). Because only living cells can regulate Ca^{2+}, we used Ca^{2+} imaging to further examine the viability of encapsulated cells. KCl and the L-type Ca^{2+} channel agonist BAY K 8664 were able to stimulate $[Ca^{2+}]_i$, whether applied into a single capsule or to the medium adjacent to a capsule (Figs. 3 and 4). Only a slight delay in the response was noted on external application. Of interest was the observation that the central cores of larger aggregates appeared to be equally responsive to stimulation but less efficient at loading the Ca^{2+} indicator, since the resting Ca^{2+} fluorescence was lower in central compared with peripheral cells. These data suggest that the cells in the central cores may be less accessible and therefore less responsive to larger molecules capable of crossing the capsule membrane. It further suggests that this potential phenomenon would become even more apparent as the cell mass expands and the aggregates enlarge.

In an earlier publication by Knaack et al. (8), βTC6-F7 cells were reported to have manifested a stable phenotype of insulin secretion in response to physiological glucose concentrations. Our results are in agreement with this previous study in that the cells were responsive to glucose; however, our studies demonstrate that there is a progressive increase in the insulin response to glucose through 16.8 mM (Fig. 2). There are several differences between the two studies, including the culture conditions, type of medium, glucose concentration, and the type of serum conditions used. In addition, we did not use the phosphodiesterase inhibitor IBMX in timed release assays. These modifications may explain the differences. In the present study, although the βTC6-F7 cells displayed a steady insulin secretion, their response to glucose stimulation appeared rather limited compared with pancreatic islets that we had previously characterized (10). In our past studies on insulin secretion from microencapsulated rat and porcine pancreatic islets, we showed that the kinetics of insulin release from the free and microencapsulated islets were very similar, thus indicating that the alginate-polylysine-alginate membrane does not constitute an impediment to the free flow of insulin. These findings were confirmed in this study by demonstrating similar patterns of insulin secretion from the free and microencapsulated βTC6-F7 cells.

In vivo, the implantation of 3×10^6 βTC6-F7 cells resulted in a long-term restoration of normoglycemia in diabetic mice. In comparison, in our past studies using islets of Langerhans, transplants of ~800 rat islets, comprising roughly 3×10^6 insulin-producing β-cells, were sufficient to reverse diabetes in mice. Unencapsulated islets implanted under the kidney capsule or into the portal vein may require fewer islets compared with an intraperitoneal transplant of immunoisolated islets (2). In our experience, at least 500 islets per mouse, comprising perhaps 2×10^6 β-cells, are necessary to sustain euglycemia. Thus, in vivo, the insulin secretion of the grafted βTC6-F7 cells appears comparable to the

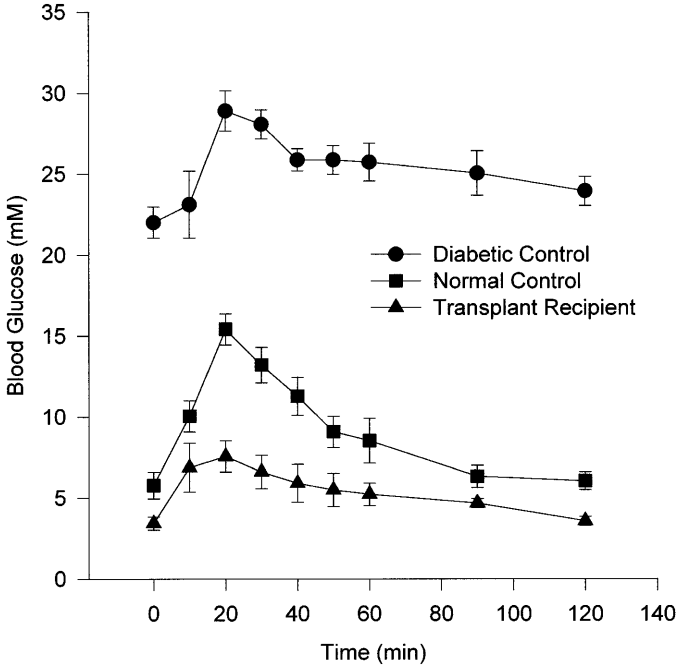

Fig. 6. Oral blood glucose tolerance test (OGTTs) of graft recipients. OGTTs were administered to transplant recipients in which normoglycemia had been established as a result of the grafts for a period of at least 1 wk. Nondiabetic animals and streptozotocin-induced diabetic mice without capsules were used as control groups. Data are presented as means ± SE of $n = 5$ animals per experimental group.

transplants of natural islet β-cells. However, as the results of the glucose tolerance tests indicate (Fig. 6), the very limited responsiveness of the βTC6-F7 cells to glucose causes the glucose clearance to be rather flat, very similar to that displayed by many insulinoma patients. This observation bears out the fact that the insulin release from the βTC6-F7 cells most likely goes on continuously, with the cells not responding to the dynamics of serum glucose concentrations. Thus, although not measured, we suspect that circulating insulin levels would be elevated in transplant recipients during the fasting state and before an OGTT.

One can only speculate about the factors responsible for the return of diabetic hyperglycemia in the recipient animals and the failure of the graft after ~55 days of normoglycemia, since in these initial studies the viability of cells after failure was not examined. Failure may have been caused by a decrease in insulin secretion or by an eventual death of the grafted cells. Also, the capsule construction is of a critical importance in this respect, as the capsules' strength determines the duration of the graft function. Similarly, imperfectly constructed capsules may limit the duration of the graft function because of surficial cell overgrowth. After the graft failure, the absolute majority of recovered capsules was found intact, with some displaying varying degrees of cell overgrowth. In these initial experiments, the reported periods of normoglycemia were shorter than those we had earlier reported for transplants of pancreatic islets, possibly indicating a limited life span of the implanted cells. Future studies could include those directed at prolonging graft survival and at enhancing the glucose responsivity of the cells.

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