Macrophage depletion improves survival of porcine neonatal pancreatic cell clusters contained in alginate macrocapsules transplanted into rats

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Abstract: Background: Macrophages can accumulate on the surface of empty and islet-containing alginate capsules, leading to loss of functional tissue. In this study, the effect of peritoneal macrophage depletion on the biocompatibility of alginate macrocapsules and function of macroencapsulated porcine neonatal pancreatic cell clusters (NPCCs) was investigated. Methods: Clodronate liposomes were injected into the peritoneal cavities of normoglycemic Lewis rats 5 and 2 days before the transplantation. Empty or NPCC-containing Ca-alginate poly L-lysine (PLL)-coated macrocapsules were transplanted into the peritoneal cavities of rats injected with either clodronate liposomes or saline. On days 7, 14 and 21, samples were evaluated by immunohistochemistry for cellular immune responses on the surface of the macrocapsules and for macrophage populations in omental tissue. To assess the function of macroencapsulated NPCCs, insulin secretory responses to glucose and theophylline were measured after capsule retrieval. Results: In saline-injected control groups, all of the empty and NPCC-containing macrocapsules were overgrown with macrophages, this being especially severe on NPCC-containing macrocapsules. In the clodronate liposomes-injected group, the majority of the empty macrocapsules were free of macrophage accumulation and the NPCC-containing macrocapsules were less overgrown than in control animals. Higher insulin responses to glucose and theophylline were observed in NPCCs retrieved from rats injected with clodronate liposomes. Conclusion: We conclude that depletion of peritoneal macrophages with clodronate liposomes improve the survival of macroencapsulated NPCCs.

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Introduction

Transplantation of porcine islet cells remains a potential treatment option for people with diabetes. Multiple sources of porcine islet tissue have been considered [1,2]; porcine neonatal pancreatic cell clusters (NPCCs) have some particularly attractive attributes, being stable in tissue culture and having considerable capacity for both growth and differentiation after transplantation [3,4]. One of the biggest problems facing the use of porcine islet tissue is its destruction by xenotransplant rejection [5]. A potential avenue has been to use immunobarrier protection, with alginate encapsulation being a commonly used method [6,7]. Unfortunately, the results have been very inconsistent, with failure being the usual outcome. In addition to an immune response stimulated by the

porcine tissue, the impurities in alginate and membrane materials can be reactive [8,9]. We have recently found that microcapsules made of highly purified alginate with a high mannuronic acid content and no poly L-lysine (PLL) coatings generate no obvious reaction after many months in the peritoneal cavity of mice [10]. In the present study, alginate macrocapsules rather than microcapsules were employed, with PLL being used as an outer coating. This formulation led to a severe reaction in Lewis rats, with the surface of the capsules being coated with macrophages. Experiments were performed to see if temporary depletion of peritoneal macrophages by clodronate liposomes would reduce the inflammation on the capsular surface and improve the survival of transplanted NPCCs contained within these macrocapsules.

Methods

Preparation of clodronate liposomes

Multi-lamellar liposomes were prepared as described previously [11]. In brief, 86 mg phosphatidyl choline and 8 mg cholesterol (Sigma, St Louis, MO, USA) were dissolved in 10 ml chloroform in a round-bottom flask and dried in vacuum at 37°C by rotary evaporation to form a thin film on the interior wall of the flask. After addition of 10 ml of phosphate buffered saline (PBS) containing 2.5 g clodronate (dichloromethylene biphosphonate, Cl_2MBP, gift of Roche Diagnostics GmbH, Mannheim, Germany), the film was dispersed into liposomes by gentle rotation. The preparation was kept for 2 h at room temperature, sonicated (50 Hz) for 3 min at 20°C and kept at room temperature for another 2 h. The liposomes were then centrifuged at $25\,000 \times g$ for 30 min to remove free clodronate and finally resuspended in 4 ml PBS. This suspension contained about 6 mg/ml of clodronate liposomes. The liposomes were kept at 4°C until injection. All liposomes were used within 1 month of preparation. On days 5 and 2 before transplantation, 1 ml of clodronate liposomes (6 mg) or saline was injected into the peritoneal cavities of normoglycemic Lewis rats using a 10-ml syringe with 23G needle.

Production of NPCCs

NPCCs were generated from neonatal pigs using a modification [4] of the technique of Korbutt et al. [3] described elsewhere. Briefly, pancreases were taken from Yorkshire pigs (1 to 3 days old, Parsons Farm, Hadley, MA, USA) with a surgery

carried out under general anesthesia (intramuscular injection of 0.15 ml Telazol (tiletamine HCL, Fort Dodge Laboratories, Ft Dodge, IA, USA) and 0.30 ml Xylaject (xylazine HCL, Phoenix Pharmaceutical, Inc. St Joseph, MO, USA). The pancreases were digested with mechanically by mincing into 1 to 2-mm^3 pieces in a 50-ml Falcon tube and then enzymatically by shaking in a water bath at 37°C followed by adding up collagenase, 5 mg/ml (collagenase P, Roche, Indianapolis, IN, USA). After filtration and two washings, the pellet was suspended in Ham's F10 media (Life Technologies Inc., Grand Island, NY, USA) supplemented with 10 mM glucose, 10 μM 3-isobutyl-L-methylxanthine, 2 mM L-glutamine, 10 mM nicotinamide, 25 mg/l of $CaCl_2 : H_2O$, 0.5% bovine serum albumin (RIA grade, A-7888, Fraction V, Sigma) and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml). NPCCs were then transferred into 150×50-mm bacteriological plates (Becton Dickinson, Franklin Lakes, NJ, USA) to be cultured at 37°C for 8 days. On day 2, there was a full change of media and on days 4, 5 and 7, half changes were performed.

Encapsulation

On day 8 after isolation, NPCCs were removed from culture plates, washed two times with Hank's balanced salt solution and once with PBS, then resuspended in 3.3% of highly purified sodium alginate (Keltone LV, provided by Dr P. De Vos) consisting of 60% of mannuronic acid and 40% guluronic acid. One milliliter of alginate solution was mixed with 3000 NPCCs. Alginate–PLL macrocapsules were made by extrusion of alginate or an alginate/NPCC suspension through a 23G needle into a 100-mM $CaCl_2$ solution. After cross-linking, the macrocapsules were incubated in a 0.1% PLL solution (MW 15 000 to 30 000; Sigma) for 10 min. An outer alginate layer was applied by incubation in a 0.3% alginate solution for 5 min. The final macrocapsules each contained 30 to 50 NPCCs and had a diameter of 1.6 ± 0.2 mm.

Transplantation and retrieval of capsules and omental samples

Empty or NPCC-containing macrocapsules (20 to 25 macrocapsules in number) suspended in 1 ml of sterile PBS were transplanted into the peritoneal cavity of normoglycemic male Lewis rats (300 g body weight, Harlan Sprague Dawley, Indianapolis, IN, USA), which had been injected 5 and 2 days previously with clodronate liposomes or saline. Macrocapsules were introduced into the peritoneal

cavity through a small abdominal midline incision using a cut 1-ml syringe. For the empty macro-capsules, there were three rats in both groups for each time point. For the experiments with NPCC containing capsules, in the clodronate liposomes group there were seven, six and four rats for days 7, 14 and 21, respectively, while in the saline-injected group there were three rats at each time point. On days 7, 14 and 21, macrocapsules were removed by peritoneal lavage using warmed Krebs–Ringer–HEPES (KRH) buffer, and biopsies were taken from the greater omentum where it was connected to the greater curvature of the stomach.

Gross assessment of capsules after retrieval

The percentage of capsules recovered was deter-mined as the number of recovered capsules divided by the number transplanted. For better visualiza-tion of the encapsulated NPCCs, dithizone (dim-etylthiocarbazone, Sigma, St Louis, MO, USA) staining was performed using a freshly prepared solution, made by dissolving 25 mg of dithizone in 9 ml of 95% ethanol with hand-shaking, followed by the addition of 250 μg of pure ammonium hydroxide. One milliliter of the dithizone–ethanol solution was added to a 60-mm Petri dish contain-ing the capsules. After 10 min, the excess dithizone was removed with several washings with KRH. To assess overgrowth, all retrieved capsules were examined with phase contrast microscopy. Bioin-compatibility of empty and NPCC-containing macrocapsules was defined as the percentage of the retrieved capsules that were overgrown. Pho-tomicrographs were made using Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA, USA) with a Sony 3CC video camera and Scan-lytics LG-3 frame grabber.

Histology and immunostaining

Five macrocapsules from each rat and omental tissue samples were snap frozen in isopentane (Fisher Scientific, Pittsburgh, PA) chilled by liquid nitrogen. The frozen tissue was kept at –80°C until sectioning. Macrocapsules were cut in a cryostat (AS 620 Cryotom, Shandon, Pittsburgh, PA, USA) at –25 to –30°C and omental tissue was sectioned with a different cryostat (CM3050-3-1-1, Leica Microsystems, Germany) at –35 to –40°C into 5-μm sections.

Pericapsular inflammatory reaction was assessed using hematoxylin and eosin, aniline blue, and immunostaining. Markers for macrophages and CD8 positive T cells included the following mono-clonal anti-rat antibodies: ED1 (2245 ID, Pharm-ingen, San Diego, CA, USA), ED2 and ED3 (MCA 342 R and MCA 343, respectively, Serotec Inc., Raleigh, NC, USA), CD4 and CD8 (2202 and 2207 ID, respectively, Pharmingen). ED1 is expressed in the cytoplasm of non-activated mac-rophages and dendritic cells [12]. ED2 is expressed on the cell surface of activated macrophages [13] and is thought to be a differentiation antigen. ED3 is expressed on the cell surface of a subpopulation of macrophages, including marginal zone macro-phages in the spleen and sub capsular sinus macrophages in the lymph nodes [13].

For immunostaining, sections were dried for 30 min at room temperature, fixed in acetone for 2 min at 4°C and washed in PBS at room temperature. After 20 min incubation in PBS containing 5% horse serum, primary antibodies were added to the sections and incubated overnight at 4°C. Endogenous peroxidase activity was blocked by a hydrogen peroxide–methanol solu-tion (1 : 500), and endogenous biotin was blocked with avidin–biotin blocking kit (VECTOR Esp Inc., Houston, TX, USA) for 30 min. Then, the sections were rinsed with PBS for 5 min, and incubated with biotinylated horse anti-mouse antibody (BA-2001, VECTOR) for 1 h. After rinsing with PBS, the avidin–biotin complex (ABC # kit KO355, horseradish peroxidase, DAKO, Carpinteria, CA, USA) was added to the slides for 30 min. After being rinsed with and kept in PBS for 10 min, slides were placed in diam-inobenzidine substrate solution for 10 min (DAB substrate kit, VECTOR) and then rinsed with distilled water. Counterstaining was performed with a Gill II hematoxylin solution for 15 s. Omission of the primary antibody was used as a control staining specificity.

The degree of overgrowth on the macrocapsules was assessed by counting the number of cellular layers and number of macrophages from at least three macrocapsules retrieved from two rats at each time point. For the assessment of inflammation, overgrowth was defined as at least one layer of macrophages on the surface of the macrocapsules. Capsules were considered to be "clean" if they contained no visible overgrowth. The numbers of macrophages were counted systematically employ-ing light microscopy (cells were counted from every other field, with the total cell number being collected from five fields using a 40× objective with a final magnification of 830×). The numbers of cell layers were also counted on the same fields. The degree of macrophage accumulation was scored on a semiquantitative basis from 0 to 3, with 0 being no cellular layer observed on any field, thus represent-ing clean macrocapsules; 1 being less than 50 cells in

each field, representing mild overgrowth; 2 being and 51 to 100 cells in each field representing moderate overgrowth; and 3 being more than 100 cells, being considered severe overgrowth. CD4- and CD8-stained cells were assessed in the same way. The presence of cell layers was scored with 0 being no cell layer on any of the sections; 1 being less than five layers representing a mild level; 2 being 6 to 10 layers representing moderate level of overgrowth and 3 being more than 10 layers representing severe overgrowth. Eosinophils were counted on H & E stained sections of macrocapsules (minimum of three macrocapsules from each rat and two rats for each time points).

For the assessment of inflammation, on each section of omentum, the number of macrophages was counted on nine representative fields at high magnification of 420× and the number was averaged for each animal. The counting was carried out on blindly selected sections from two rats (in most cases) for each time point. The degree of macrophage accumulation per animal was scored from –1 to 3, with –1 being less than 20 macrophages per field representing depletion of macrophages; 0 being 21 to 50 cells, the baseline presence of macrophages in pre-transplant samples. Scores of 1 to 3 were considered to represent increases in the number of macrophages, with 1 (mild) being 51 to 80 cells per field, 2 (moderate) being 80 to 100 cells and 3 (severe) being defined as more than 100 cells.

In vitro insulin release from retrieved macroencapsulated NPCCs

The functional responsiveness of macroencapsulated NPCCs (n = 3 rats for each time point) was studied in vitro with sequential 12 h static incubations after transplantation. Triplicate samples of two macrocapsules from each rat were transferred into wells of 24-well Falcon culture plate (Becton Dickinson). Prior to glucose stimulation, the macrocapsules were washed three times and incubated for 3 h in RPMI media (Life Technologies, Inc, Grand Island, NY, USA) containing no glucose and supplemented with 10% neonatal calf serum. The secretion studies were performed at 37°C in 1.5 ml of Ham's F10 media supplemented with 10% neonatal calf serum and different glucose concentrations. After incubation of capsules in media containing 5.6 mM glucose for 12 h, capsules were exposed to 16.7 mM glucose for 12 h and then 16.7 mM glucose + 10 mM theophylline for an additional 12 h. At the end of each 12 h incubation period, supernatant media were collected in 1.5 ml Eppendorf tubes and kept at –20°C until measurement of insulin, which was performed with a radioimmunoassay kit (Linco Research Inc.,

St Charles, MO, USA), with samples measured in duplicate. The insulin responses to glucose and glucose + theophylline are expressed as ng/macrocapsule/12 h.

Statistical analysis

Results are presented as the mean ± SEM. For statistical evaluation, the unpaired Student's *t*-test was performed. Differences were considered to be statistically significant if the P-value was less than 0.05.

Results

Characteristics of macrocapsules recovered after transplantation

The efficiency of recovery of both empty and NPCC-containing macrocapsules was usually in the range of 75 to 95% but were less than 50% because of breakage of the capsules in one experiment (Table 1).

Empty macrocapsules

In the saline-injected control group, all macrocapsules were overgrown at all time points, whereas in the rats treated with clodronate liposomes, the vast majority of empty macrocapsules were free of overgrowth at all time points (Figs 1 and 2). The cellular infiltration on the macrocapsules from control rats was characterized by increasing thickness of the cell layers; scores were (1, 1) at day 7, (2, 2) at day 14 (Fig. 4, lower panel) and (3, 3) at day 21 (data not shown). The majority of mononuclear cells on the macrocapsules expressed macrophage-specific phenotypes (ED1, ED2 and ED3). For CD4 cells, there was mild infiltration (1, 1) at day 7

Table 1. Evaluation of empty and NPCC-loaded macrocapsules after retrieval

	Retrieval (%)		
	Clodronate	Control	P
Day 7			
Empty (n = 3)	80 ± 10	87 ± 8	n.s.
NPCCs (n = 7)	74 ± 7	76 ± 5	n.s.
Day 14			
Empty (n = 3)	92 ± 8	77 ± 7	n.s.
NPCCs (n = 6)	50 ± 11	70 ± 10	n.s.
Day 21			
Empty (n = 3)	95 ± 2	82 ± 5	0.05
NPCCs (n = 4)	93 ± 11	86 ± 11	n.s.

Alginate–PLL macrocapsules were retrieved from the peritoneal cavity of clodronate or saline-injected normoglycemic Lewis rats. The efficiency of recovery of both empty and NPCC-containing macrocapsules was usually in the range of 75–95%, except for low recovery on day 14 because of breakage in one set of experiment.
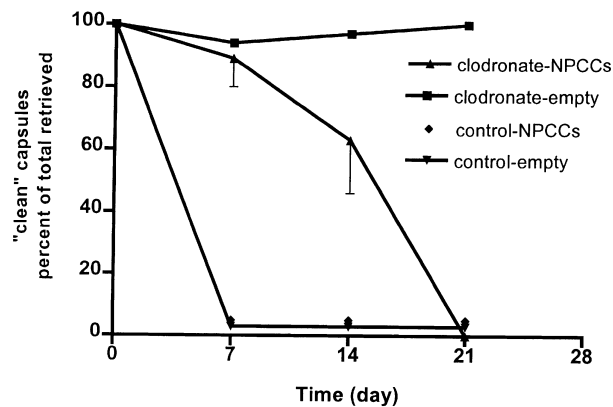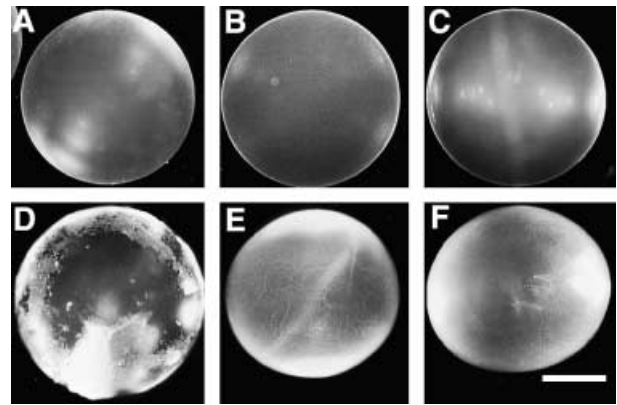
Fig. 1. Percentage of "clean capsules" recovered from clodronate liposomes- and saline-injected control rats. In the control groups, the capsules were completely overgrown at all time points after 0 day. In the clodronate liposomes-injected group, the empty capsules were mainly clean (94% at day 7, 97% at day 14 and 100% at day 21). The porcine neonatal pancreatic cell cluster containing macrocapsules in this group were less overgrown than in the control group at days 7 and 14, with clean capsule recovery being 89 and 63%, respectively, but by day 21 they were completely overgrown.

Fig. 2. Representative empty capsules from clodronate liposomes- or saline-injected rats. "Clean" capsules recovered from clodronate liposomes-injected rats are shown on the upper row and overgrown macrocapsules from saline-injected rats are shown on the lower row. Images A and D were taken at day 7, B and E at day 14, and C and F at day 21, after retrieval. The surface of overgrown macrocapsules (D, E, F) had an irregular, granular and opaque appearance. Magnification bar = 500 μm.

and moderate to severe infiltration (2, 3) at day 14. For CD8 cells, there was a mild infiltration (1, 1) at all time points (data not shown). Although double staining was not performed, the CD4-positive cells had a macrophage-like phenotype and CD8-positive cells appeared to be T cells. On H & E stained sections, scattered eosinophils (less than five cells on each section at all time points) were found.

NPCC-containing macrocapsules

In saline-injected control rats, NPCC-containing macrocapsules were even more severely (3, 3) overgrown with macrophages using the three markers at all time points. This overgrowth consisted of thick cell layers, this thickness being scored as (2, 3) at day 7 and (3, 3) at days 14 and 21 (up to 30 layers of cells), considerably more severe than what was seen on the empty-macrocapsules (Figs 1, 3 and 5). Infiltration of CD4 cells was mild (1, 1 at day 7) to severe (3, 3 at days 14 and 21), while accumulation of CD8 cells on these capsules was mild (1, 1 at each time points). More eosinophils (more than 20 on each section) were found in the cellular infiltrate on NPCC-containing capsules than on empty capsules. In rats treated with clodronate liposomes, the inflammatory overgrowth was much reduced at days 7 and 14, being present on only 11 and 37% of the capsules, respectively (Fig. 1). Nonetheless, by day 21, the effect of the clodronate liposomes was lost and NPCC-containing capsules were completely overgrown with macrophages.

Macrophage populations in omental samples

Rats with empty macrocapsules

Omental samples from saline-injected rats showed clear but modest increases in macrophage infiltration at days 7, 14 and 21 using all three markers, with it being impossible to see clear differences between ED1, ED2 and ED3 (Table 2, Fig. 6). Thus, even empty alginate–PLL macrobeads elicited an immune reaction in the omentum. In the rats injected with clodronate-liposomes, the macrophage infiltration of the omentum was not increased at any of the subsequent time points, remaining at a level indistinguishable from the baseline values.

Rats with NPCC-containing macrocapsules

The omental samples from saline-injected rats showed a very severe infiltration of macrophages at day 7 with there being nodular infiltration of ED1, ED2 and ED3 stained cells (Table 2, Fig. 7). This infiltration became moderate at days 14 and 21, with no clear differences in the relative frequencies of the different macrophage phenotypes, but the infiltration remained considerably more severe than for the empty capsules. The clodronate liposomes produced a marked inhibition of macrophage infiltration on the NPCC-containing capsules on day 7, but by day 14 the severity of omental infiltration returned to that seen in the saline injected group. At 21 days, variability was seen, so that baseline infiltration was found in the omentum of some rats while others had severe infiltration.
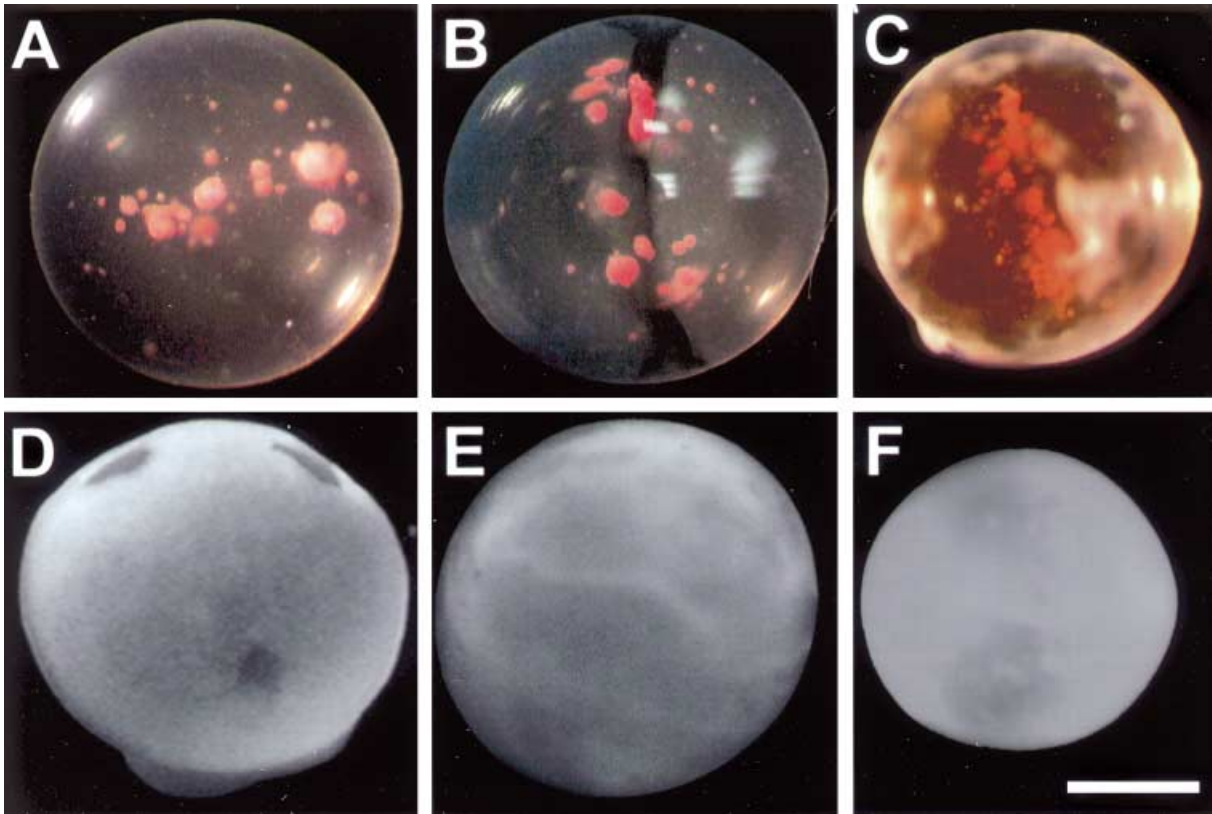
Fig. 3. Representative porcine neonatal pancreatic cell clusters (NPCCs) containing macrocapsules retrieved from clodronate liposomes- (upper row) or saline-injected (lower row) rats. Images A and D were taken at day 7, B and E at day 14, and C and F at day 21. NPCCs inside the capsules were stained with dithizone. There was much less overgrowth on the macrocapsules retrieved from the clodronate liposomes- than saline-injected rats, but a cellular reaction could be seen at 21 days. The cellular overgrowth was accompanied by shrinkage of the capsules, as suggested by a smaller diameter of about 1.2 ± 0.2 mm. Magnification bar = 500 μm.

Insulin secretion in vitro from retrieved encapsulated NPCCs

Insulin secretion in response to high glucose and the combination of high glucose and theophylline was about the same from macroencapsulated NPCCs retrieved 7 days after transplantation in the control and clodronate liposomes-injected groups (Fig. 8). However, at 14 and 21 days, insulin responses from NPCCs contained in severely overgrown capsules reduced, even becoming undetectable at day 21. In contrast, with macrocapsules retrieved from clodronate liposomes-treated rats the insulin responses to glucose, and glucose plus theophylline were higher. This increase was probably because of both maturation of the NPCCs inside the capsules and the reduction in cellular overgrowth as a result of the clodronate liposomes treatment.

Discussion

In this study, it was found that intraperitoneal injections of clodronate liposomes were able to inhibit macrophage accumulation on the surface of empty and NPCC-containing alginate–PLL macrocapsules. In addition, it appeared that this maneuver promoted the function of NPCCs inside the capsules, as indicated by preservation of stimulated insulin secretion in vitro after the capsules were retrieved. As judged by the development of cellular overgrowth 21 days after the transplant (23 days after the last clodronate treatment), the limited duration of the treatment is evident. This approach was not attempted in diabetic Lewis rats, but the preserved insulin secretion from porcine β-cells in the capsules suggests that macrophage depletion could have a temporary beneficial effect in promoting normalization of glucose levels.

Probably, the most convincing demonstration that an immunobarrier approach can provide protection against xenorejection was shown with a vascularized hollow fiber device that protected adult porcine islets in dogs for many months [14]. The challenge may be more difficult for micro or macrocapsules made from gel materials such as agarose or alginate because of differences in diffusion characteristics from a vascularized
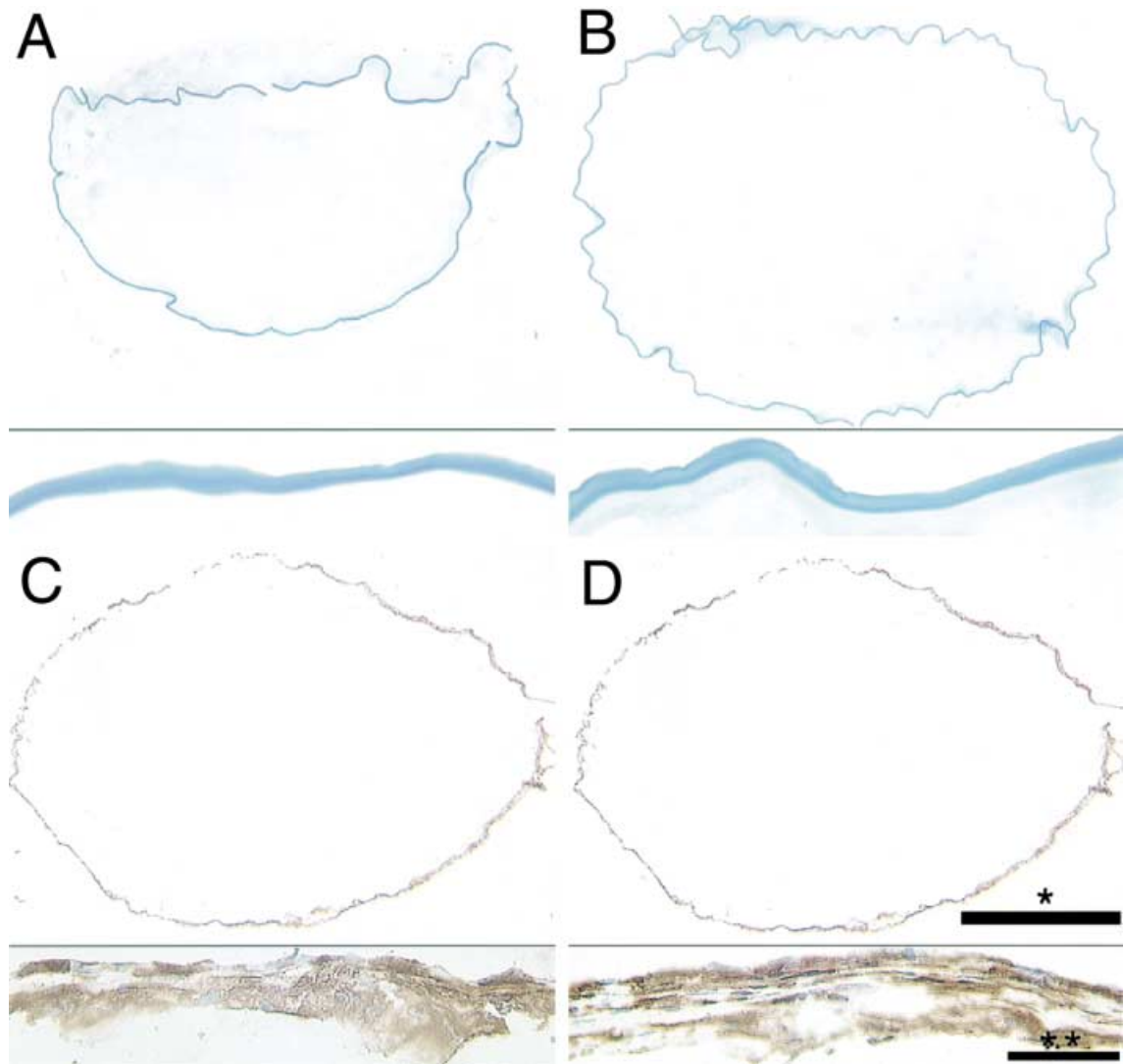
Fig. 4. Immunostaining of empty macrocapsules. Sections of non-overgrown macrocapsules (days 7 and 14) from clodronate liposomes-injected rats are in the upper panels (A and B) and overgrown macrocapsules from saline-injected rats are in the lower panels (C and D). On each panel, the upper row images were taken at low magnification (*magnification bar = 500 μm) and lower images at high magnification (**magnification bar = 100 μm). Non-overgrown macrocapsules (clodronate liposomes group) were stained with aniline blue, which stained the outer poly-L-lysine coating of the alginate capsules. Macrocapsules from saline-injected rats were stained with the macrophage specific ED2 antibodies, showing the presence of several layers of macrophages.

device. Nonetheless, some successes have been reported with prolonged survival of porcine insulin-producing cells in mice [15,16]. Rats are thought to provide a stronger immune response than mice, but some success in rats has been reported with alginate microcapsules [7], polyethylene glycol microcapsules [17] and agarose macrocapsules [18]. An impressive result published several years ago showing that porcine islets in alginate–PLL microcapsules could prolong normalization of glucose levels in spontaneous diabetic monkeys has not yet been confirmed [19]. In spite of the lack of reproducible success at the present time, it seems possible that capsules made with improved materials containing porcine islet cells could at some point be used for clinical transplants in humans with diabetes.

The current study provides new information about the tissue reaction elicited by empty and NPCC-containing alginate–PLL macrocapsules and how it is influenced by clodronate liposomes. Previous studies have found macrophage accumulation on the surface of transplanted immunobarrier membranes [20–22]. Some of the components that can lead to tissue reactions include impurities in the alginate, endotoxin content, responses to the PLL and immunological reactions to the contained islet material. There have been debates about the bioincompatibilty of alginate with different composition [23–25], but more recently workers have
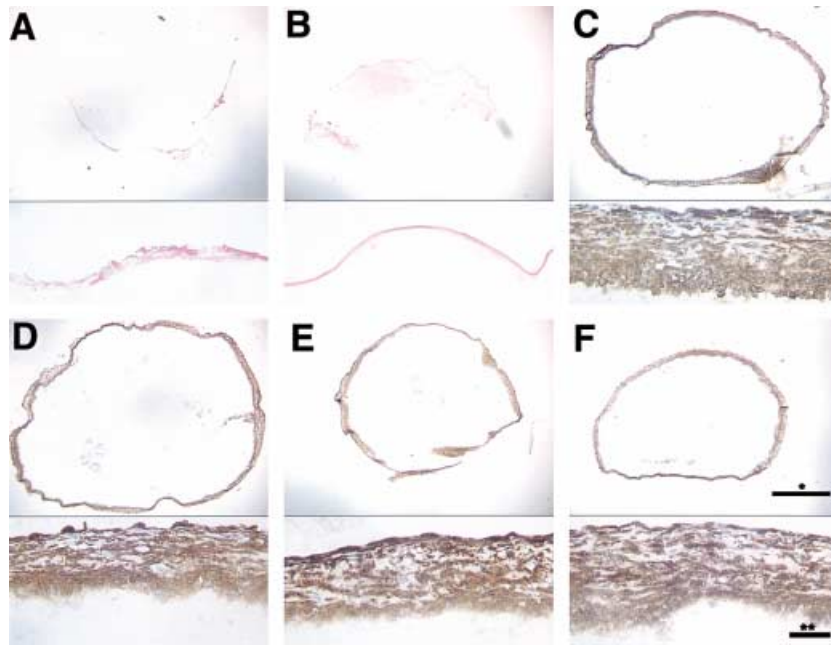
Fig. 5. Porcine neonatal pancreatic cell cluster containing macrocapsules were stained with eosin and the macrophage specific ED2 antibodies. Representative macrocapsules were recovered from clodronate liposomes-injected group (upper panel, A, B and C, representing days 7, 14 and 21, respectively) and saline-injected group (lower panel, D, E and F, representing the same three time points). Capsule sections are shown at both low (upper row images, *magnification bar = 500 μm) and high magnification (lower row images, *magnification bar = 100 μm). For the clodronate liposomes-injected group, the capsules were largely free of infiltrate on days 7 and 14, but by day 21 had become heavily covered with macrophages, as shown by the brown staining. In the saline-injected control group, a thick layer of inflammatory cells could be seen at all three time points.

Table 2. Macrophage accumulation in omentum samples taken from clodronate-liposomes or saline-injected animals at days 7, 14 and 21
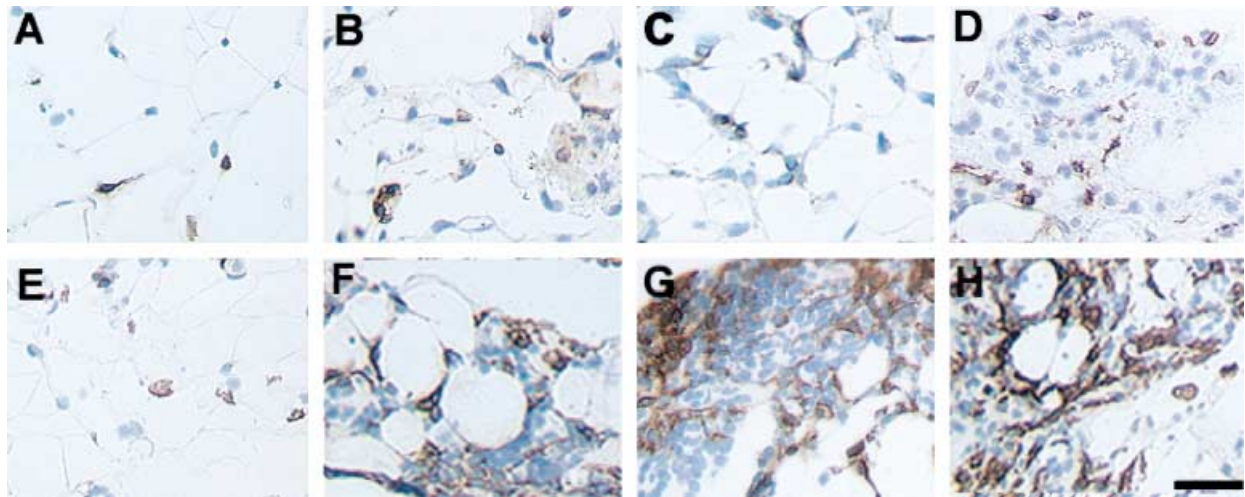
Time	Macrocapsule	Clodronate-liposomes			Saline		
		ED1	ED2	ED3	ED1	ED2	ED3
Day 0		1, 0, 1	1, 0, 0	1, 1, 1	−1, −1, 0, 0	1, 1, 1, 1	−1, −1, 0, 0
Day 7	Empty	0, 0	1, 1	−1, −1	1, 2	2, 2	1, 2
	NPCCs	−1, 0	1, 1	−1, −1	3, 3	3, 3	3, 3
Day 14	Empty	0, 0	−1, 1	−1, 0	3, 1	1, 2	1, 2
	NPCCs	2, 1	3, 3	2, 2	2, 2	2, 2	2, 2
Day 21	Empty	−1	1	−1	1, 2	0, 2	1, 1
	NPCCs	−1, 3	1, 3	−1, 3	2, 2	1, 2	2, 3

The numbers of positive cells were counted in nine fields at 420× and meaned for each animals; the value for each rat is presented in a semiquantitative manner. A graft score of −1 represents depleted cells, 0 represents a pre-transplantation baseline macrophage appearance in controls, 1 represents a mild increase in macrophage infiltration, 2 represents a moderate increase in macrophage infiltration and 3 means a severe increase in macrophage infiltration. In groups with empty macrocapsules, omental samples taken from clodronate-injected rats showed baseline level of macrophages on day 0 and maintained this level on days 7, 14 and 21. In the saline-injected rats with empty capsules the omental samples showed a modest increase in macrophage infiltration. In NPCC-containing macrocapsule groups omental samples from saline-injected rats showed severe macrophage infiltration at day 7, which decreased to a moderate level by days 14 and 21. In contrast, the samples from clodronate-injected rats showed no increase or even depletion at day 7, but moderately severe infiltration returned at day 14 and variable degrees of infiltration were found at day 21.

found that both types of alginate (high content of mannuronic acid or high content of guluronic acid) are biocompatible if highly purified [26] and used without PLL coating [27]. Because of concerns about the reactivity of PLL, Duvivier-Kali et al. used microcapsules made of highly purified alginate with no PLL coating and found virtually no reactivity for longer than 300 days in mice [10]. These microcapsules were able to protect against both autoimmunity and alloreactivity in mice, but it is not yet clear how effective they will be for the protection of xenografts. In the present study, a different preparation of alginate was used which may not have been as biocompatible, but the PLL could have been the major contributor the reaction. Based on pilot experiments in rats suggesting poor capsule stability as evidenced by a high breakage rate of uncoated macrocapsules (data not

Fig. 6. Immunostaining of representative samples of omentum taken from rats transplanted with empty macrocapsules. The macrophage specific marker ED2 was used for staining. Tissue samples from rats injected with clodronate liposomes were taken at days 0, 7, 14 and 21 (upper panels, A, B, C and D, respectively) and from saline-injected rats taken at the same time points (lower panels, E, F, G and H). It is clear that the empty capsules alone elicited a strong macrophage response in the omentum of the control group, and this response was markedly abated by the clodronate liposomes treatment. A similar pattern of staining was found on sections stained with ED1 and ED3 (see Table 2). Magnification bar = 100 μm.
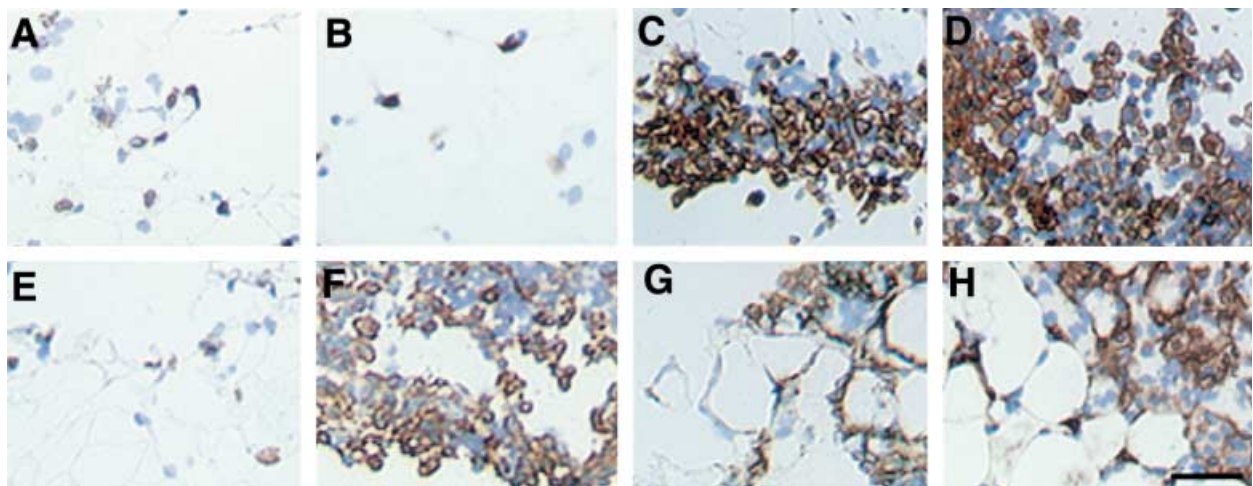
Fig. 7. Immunostaining of representative samples of omentum taken from rats transplanted with porcine neonatal pancreatic cell clusters containing macrocapsules. The macrophage specific marker ED2 was used for staining. Tissue samples from rats injected with clodronate liposomes were taken at days 0, 7, 14 and 21 (upper panels, A, B, C and D, respectively) and from saline-injected rats at the same time points (lower panels, E, F, G and H). The macrophage infiltration of the omentum was much less severe in the rats injected with clodronate liposomes, although increased infiltration was seen starting at day 14. A similar pattern of staining was found on sections stained with ED1 and ED3 (see Table 2). Magnification bar = 100 μm.

shown), we decided to use PLL-coated macrocapsules for the present experiments. In an earlier study, however, we found that empty macrocapsules made with barium cross-linked alginate and no PLL had reasonably good biocompatibility [28]. Another variable is the size of the capsules, as it is possible that large capsules of 1.5 mm or larger diameter may lead to different reactions than capsules smaller than 1 mm.

Although the empty macrocapsules developed overgrowth with macrophages and other cellular elements, the reaction was worse with more intense T cell involvement when NPCCs were contained in the capsules. This result shows how a xenograft reaction from encapsulated islets has a separate effect, which may work additively or even synergistically with the reaction against the biomaterials. Although the xenograft reaction against non-encapsulated porcine tissue has been described in detail and is very complex, little is known about the responsible antigens [29,30]. Presumably, antigens that leaked into the peritoneal cavity caused the
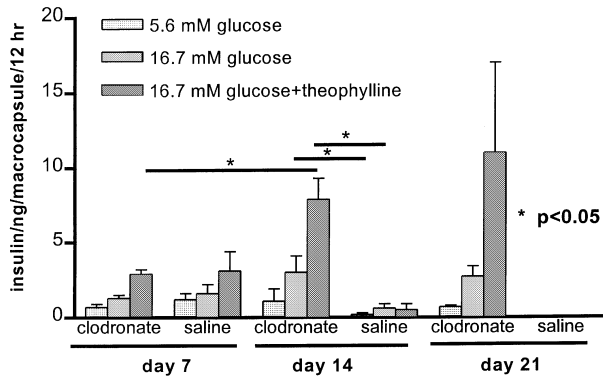
Fig. 8. Insulin secretion during static incubations of porcine neonatal pancreatic cell clusters (NPCCs) contained in macrocapsules after retrieval from transplanted rats. Triplicate samples of two macrocapsules containing NPCCs from each of the animals were sequentially incubated for 12-h periods with 5.6 mM glucose, 16.7 mM glucose, and finally 16.7 mM glucose + 10 mM theophylline, all contained in Ham's F10 media. Insulin output is expressed as mean ± SEM. In the control group with severely overgrown macrocapsules insulin output fell off to undetectable levels by day 21, while insulin response to glucose (clodronate vs. control at day 14, $P < 0.05$), and glucose + theophylline (compared with day 7 clodronate and day 14 control macrocapsules, $P < 0.05$) were higher in the macrocapsules retrieved from clodronate liposomes treated rats.

severe reaction to the NPCCs in the present study. The barrier produced by the capsule does not allow direct access of the host immune cells to the NPCCs inside, but inflammatory cells are found on the outside the capsules and in tissue samples taken from host omentum. The reaction in the omentum is presumably a tissue response to released xeno-antigen, but the mechanisms responsible for the attachment of macrophages to alginate are much less well understood.

The features of immune response in rats transplanted with fetal porcine pancreatic tissue have been described by Wallgren et al. [31]. This reaction consists of mainly ED1 and ED2 macrophages, and CD4 positive macrophages, with fewer CD2 and T cell receptor α/β positive T lymphocytes. The most interesting finding was that the majority of CD4 positive cells were defined as macrophages because of histo-morphological similarities to ED1 positive macrophages. Furthermore, very few T cells stained with TCR α/β and CD2 markers were found. This information was consistent with another study carried out by Candidas et al. with rats transplanted with guinea pig heart [32], in which they found T cell-independent macrophage and NK cell infiltration. However, in primate studies carried out in *cynomolgus* monkey transplanted with fetal porcine islet-like cell clusters, there was a dominance of cytotoxic T lymphocyte [33]. In a recent report,

Krook et al. have shown that xenorejection developing in rats transplanted with fetal porcine islet cell clusters, is characterized by very strong macrophage activation, with an early Th1 activation followed by a late Th2 response. These results were obtained with the combined use of measurement of cytokine levels and immunostaining [34]. Eosinophils are well described elements of the xenorejection reaction and the fact that fewer eosinophils were seen after macrophage depletion raises questions about how macrophages might promote the attraction of these cells. Although the presence of eosinophils is of interest, questions have been raised about whether the accumulation of eosinophils is essential for xenorejection [35]. Fritschy et al. [20] reported similar histological appearance of macrophage accumulation on alginate microcapsules that were either empty or contained adult porcine islets.

The decline of insulin secretion from the retrieved NPCC-containing overgrown macrocapsules of the control group suggested death of the insulin producing cells. Although it is possible that cells were healthy and that insulin could not be released through the cellular coating of the capsules, the more likely explanation is that most of the cells had died by the effects of toxic cytokines released from surrounding inflammatory cells and by the oxygen consumption of the cells coating the capsules, which would have allowed oxygen to be stolen from the cells in the center of the capsule. It is noteworthy that the insulin secretion from the capsules of the clodronate liposomes treated group actually increased over the 14-day period. Not only was it possible for insulin to be released through whatever overgrowth was present, but it also seems likely there was maturation of the NPCCs within the capsules. It has been clearly shown that β-cell expansion occurs when NPCCs are transplanted under the kidney capsule of nude mice [3,4,36]. Moreover, it has been shown that NPCCs can mature when contained in microcapsules in the peritoneal cavity [37].

Another approach to improving the survival of microencapsulated rat islets is to using immunosuppressive drugs to diminish macrophage-mediated interleukin 1-β levels [38]. Temporary macrophage depletion with gadolinium and clodronate has been shown to prolong the survival of allogeneic islets in rats [39] and xenogeneic islets in Streptozocin (STZ) diabetic mice [40]. It has been shown that rat ED1 omental macrophages are repopulated at 1 week, whereas ED2 and ED3 macrophages are repopulated 3 to 4 week after the intraperitoneal injection of clodronate liposomes [13]. Once differentiation and activation occurs,

ED1 cells differentiate into ED2 and ED3 cells, these markers being indicative of maturation [41]. In our study, however, it was not possible to show any clear differences in macrophage phenotype during omental depletion or repopulation after injection of clodronate liposomes.

In conclusion, these data indicate that temporary macrophage depletion reduces the inflammatory reaction on the surface of alginate–PLL macrocapsules and prolongs survival of NPCCs contained within the capsules. Macrophage depletion could become one of many tools used to protect xenografts from immune destruction.

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