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Microencapsulated islet grafts in the BB/E rat: a possible role for cytokines in graft failure

D. R. Cole[1], M. Waterfall[1], M. McIntyre[2] and J. D. Baird[1]

[1] Metabolic Unit, University of Edinburgh Department of Medicine and
[2] Department of Pathology, Western General Hospital, Edinburgh, UK

Summary. Alginate-polylysine microencapsulation has been proposed as a method of protecting transplanted pancreatic islets against immunological attack. Using this technique, prolonged graft survival has been reported in some diabetic animals. However, in the spontaneously diabetic insulin-dependent BB/E rat we found that intraperitoneal implantation of microencapsulated islets had only a short-lived effect on hyperglycaemia. Recovered microcapsules (both those implanted empty and containing islets) were surrounded by a foreign body type cellular overgrowth and, although many capsules remained intact, encapsulated islets were observed to be disintegrating. Loss of Beta cells was confirmed by immunohistology. Various polymer materials used in artificial membranes have been shown to activate macrophages involved in foreign body reactions and induce synthesis of interleukin-1β, a known Beta-cell toxin. Reduced secretion of insulin and progressive islet damage (indicated by a significant reduction in residual islet insulin and DNA content) were demonstrated when microencapsulated islets were incubated with interleukin-1β in vitro for 9 days. Similar effects were seen following exposure to a combination of gamma interferon and alpha tumour necrosis factor. Successful use of microencapsulation in islet transplantation depends upon the development of biocompatible membranes. The exclusion of smaller molecules, such as cytokines, which may be involved in foreign body mediated damage and microencapsulated islet graft rejection, could also be important.

Key words: Microencapsulation, interleukin-1β, pancreatic islets, biocompatibility, transplantation, diabetes mellitus.

Microencapsulation of pancreatic islet grafts has been proposed as an alternative to lifelong immunosuppression for prevention of graft rejection and autoimmune destruction. It has been reported that microencapsulated islet allografts and xenografts have functioned well for several months in some, though not all, laboratory rodents with chemically-induced [1, 2] and spontaneous [3] diabetes mellitus. The aim of this study was to identify factors associated with failure of microencapsulated islet grafts.

First, the function of intra-peritoneal alginate-polylysine-alginate microencapsulated allografts was assessed in BB/E rats with established, spontaneous, insulin-dependent diabetes. Control diabetic animals were implanted with empty microcapsules. Microcapsules were recovered for histological examination by peritoneal lavage at the time of graft failure in experimental animals and at comparable time points from control animals.

In addition, we have investigated in vitro the protection afforded by the membrane against cytokines known to be produced by activated inflammatory cells. Various biomedical polymers have been shown to activate macrophages involved in foreign body reactions and stimulate interleukin-1β (IL-1β) production [4]; IL-1β can both suppress insulin secretion and destroy islet tissue in a dose- and time-dependent manner [5, 6], and other cytokines (including the combination of tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ) [7]) have also been found to be toxic to islets.

Materials and methods

Pancreatic islets were isolated from male Wistar Furth rats (200–250 g body weight) by collagenase digestion using an intraductal injection technique and Dextran density gradient separation [8]. Pooled islets from 8–10 animals (average yield approximately 3,000 islets) were cultured in supplemented medium (RPMI 1640 with 10% fetal calf serum, 20 mmol Hepes, 2 mmol L-glutamine, penicillin 100 U/ml and streptomycin 0.1 mg/ml) (Gibco Ltd., Paisley, Scotland, UK) for 48–72 h. Islets were microencapsulated using the alginate-polylysine-alginate method originally described by Sun and co-workers [2, 9]. To summarise, islets were suspended in a sterile filtered solution of 1.5% weight/volume sodium alginate (Kelco Gel LV, Kelco, New Jersey, USA) in a solution of 150 mmol/l sodium chloride (normal saline) at a density of about 2,000/ml and then drawn up into a 10 ml plastic syringe. Droplets were formed by

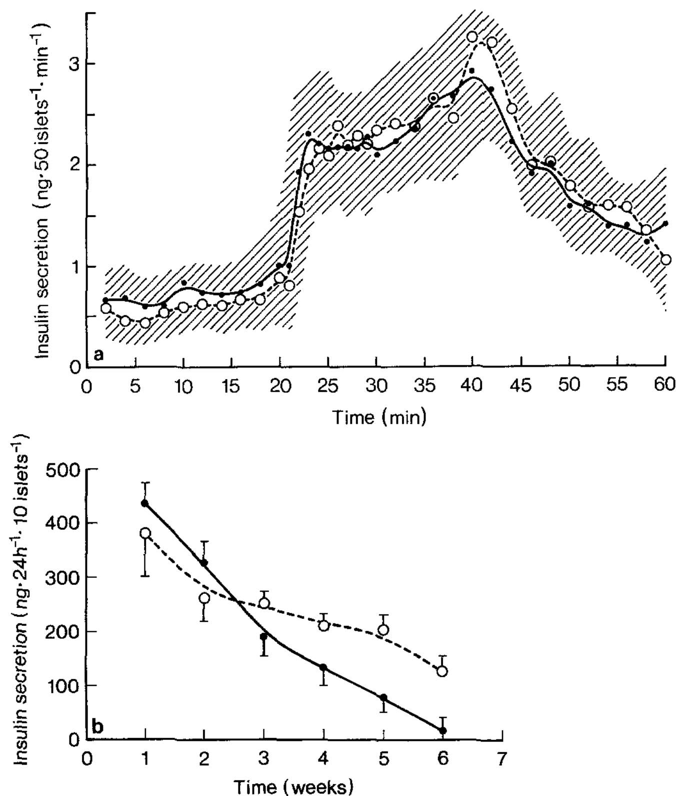

Fig. 1. (**a**) Perifusion of microencapsulated isles (○) compared to free isles (●). Glucose concentration of perifusate from 0–20 min, 2.7 mmol/l glucose; 20–40 min, 16.7 mmol/l; 40–60 min, 2.7 mmol/l. Perifusion was performed 24–48 h after microencapsulation to allow recovery from this procedure. (**b**) Static insulin release from microencapsulated islets (○) compared to free islets (●) over a 6-week incubation period in tissue culture

extrusion through a stainless steel needle surrounded by a purpose built machined air jacket, and collected in a solution of 100 mmol/l calcium chloride. Upon contact the droplets form solid beads which gravitate to the bottom of the tube. After washing in 0.1% 2-N-(cyclohexylamino)-ethane-sulphonic acid (CHES) buffer pH 8.4 (Sigma Chemical Company, St. Louis, Mo., USA) the solid beads were coated with poly-1-lysine, molecular weight 17,000 (Sigma Chemical Company) as a 0.05% solution in normal saline for 10 min followed by a further wash in CHES buffer. After a final coating with 0.15% sodium alginate in normal saline for 4 min the microencapsulated islets were suspended in 55 mmol/l sodium citrate buffer pH 7.4 for 6 min, washed and then maintained in culture medium (as above) in a 37 °C incubator with 5% CO_2 and fully humidified atmosphere for 2 days prior to implantation. Microcapsules were approximately 600 μm diameter and perfectly spherical as viewed under a low power stereomicroscope.

Islet insulin response to glucose challenge was confirmed following microencapsulation and compared with free islets using a multichannel perifusion system (n = 6) [10]. To assess function over the longer term, quadruplicate groups of 10 microencapsulated or free islets (n = 6) were maintained in 1 ml supplemented culture medium (as above) using multiwell plates and insulin release into the medium measured weekly for 6 weeks, aliquots being taken and immediately frozen to − 70 °C for later insulin assay. Culture medium was renewed every 3 days during the incubation period.

Size profiles of microencapsulated islets (islet diameter) were measured using a stereomicroscope and calibrated graticule from 3 randomly selected samples of 100 microencapsulated islets from each batch.

Spontaneously diabetic male BB/E rats aged 180 to 270 days (diabetes onset between 80 and 100 days) and maintained on daily injections of insulin were used in these experiments. Insulin injections were discontinued 24 h before microcapsules (either empty or containing islets) were suspended in physiological saline and placed in the peritoneal cavity using a 16 gauge butterfly cannula inserted into the midline through the abdominal wall under light halothane anaesthesia. Five animals were each implanted with approximately 3000 microencapsulated islets and a further five with an equivalent number of empty microcapsules. To compare function with that in a non-autoimmune model equivalent grafts were implanted into male Wistar rats of similar age made diabetic one week previously by intravenous streptozotocin, 45 mg/kg body weight administered in citrate buffer pH 4.5 (citric acid 2.1 g, molar sodium hydroxide 20 ml made up to 100 ml with sterile distilled water). Animals were assessed and weighed daily. Blood glucose was measured by BM-test 1–44 glycemie strips and Reflolux II meter (Boehringer, Mannheim, Germany) on blood samples obtained by tail tipping at 10.00 hours on the day of transplantation and daily thereafter as shown.

Microcapsules were recovered by peritoneal lavage through a small midline laparotomy incision following graft failure (defined as two consecutive blood glucose readings of > 10 mmol/l) in animals receiving microencapsulated islets and on the development of severe metabolic decompensation in those receiving empty microcapsules. Recovered microcapsules were fixed in formalin overnight prior to paraffin embedding, sectioning and staining.

Sections (4 μm thick) were stained with haematoxylin and eosin. Further sections were immunostained using a double staining technique. Mouse anti-porcine insulin monoclonal antibody (Scottish Antibody Production Unit, Carluke, Scotland, UK) diluted 1/100 in Tris-buffered saline was applied for 30 min and then layered with

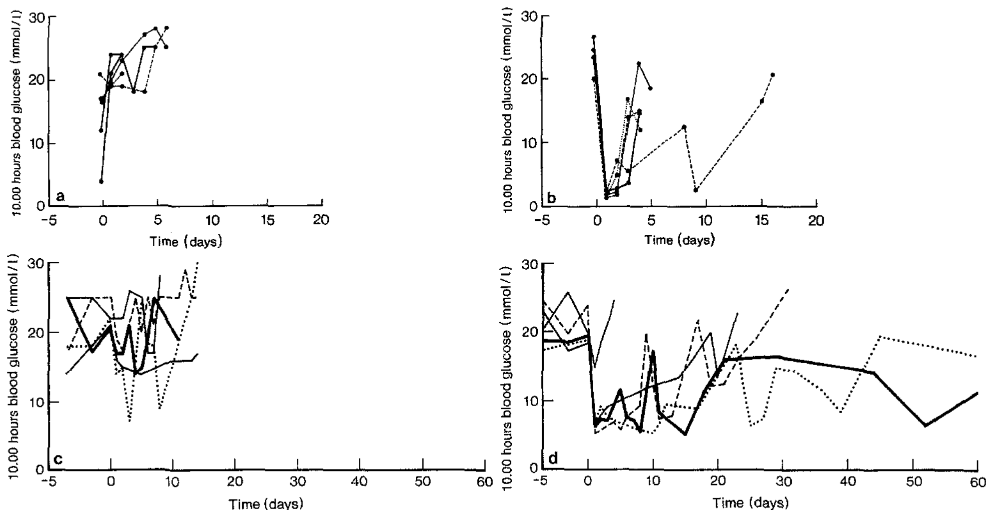

Fig. 2 a–d. Blood glucose measured at 10.00 hours in (**a**) control BB/E rats (**b**) transplanted BB/E rats (**c**) control streptozotocin Wistar rats (**d**) transplanted streptozotocin Wistar rats. For all groups n = 5. Day 0 indicates day of implantation

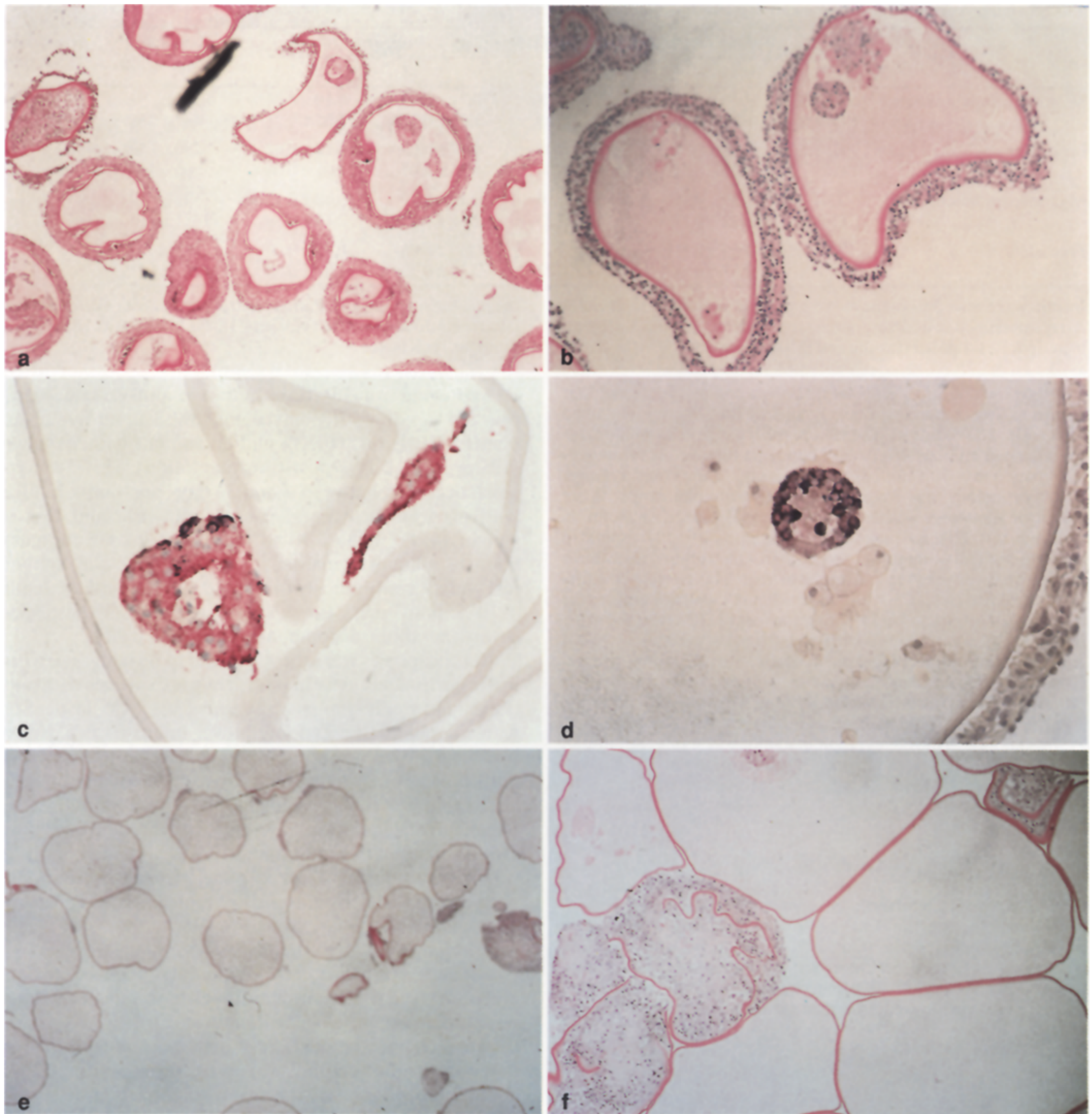

Fig. 3a–f. In vivo studies: (**a, b**) Microencapsulated islets recovered from a BB/E rat showing islet degeneration within overgrown capsules. Original magnification × 12.5 and × 30 respectively. (**c**) Microencapsulated islet after 2 weeks tissue culture immunostained for insulin (red) and glucagon (blue). Original magnification × 80. (**d**) Recovered microencapsulated islet recovered 2 weeks after implantation similarly immunostained. Original magnification × 80. (**e**) Empty microcapsules recovered 2 weeks after implantation into a streptozotocin diabetic Wistar rat. Original magnification × 12.5. (**f**) Microencapsulated islets recovered 4 weeks after implantation into a streptozotocin diabetic Wistar rat. Original magnification × 30. Note: distortion of the capsule membrane is due to fixation

biotinylated donkey antibody directed against the first antibody (Amersham International, Amersham, Bucks, UK) diluted 1/200 followed by streptavidin-alkaline phosphatase (Dako Ltd., High Wycombe, Bucks, UK) diluted 1/300, each applied for 30 min. Incubation with a substrate solution was followed by application of prediluted rabbit anti-human glucagon polyclonal antibody (Ortho, High Wycombe, Buck, UK) and the above-amplification steps repeated. All steps were separated by 5-min incubations in Tris-buffered saline. Substrates used were Fast Red and Fast Blue. Finally the sections were mounted in neutral glycerine jelly.

For in vitro studies, free or microencapsulated islets were transferred to a multiwell plate in groups of 15 per well, suspended in 1 ml of supplemented media (as above). The media was changed after 3 days incubation. A cytotoxic dose of 500 picograms of recombinant human IL-1β in RPMI (Glaxo Molecular Biology SA, Geneva, Switzerland) with 10% fetal calf serum (total volume 20 μl) was

Table 1. Effects of cytokines on free and microencapsulated islets in vitro

		Insulin secretion ((ng · 15 islets^{-1} · 72 h^{-1}) × 10^3) Time (days)				Residual Insulin (ng)	Residual DNA (ng)
		0	3	6	9		
Microencapsulated islets	IL-1β	2.10 ± 0.20	0.80 ± 0.08	0.21 ± 0.03	0.10 ± 0.04	391 ± 57	328 ± 70
	C	1.81 ± 0.20	1.68 ± 0.25	1.24 ± 0.21	1.05 ± 0.21	687 ± 65	654 ± 128
Free islets	IL-1β	2.26 ± 0.27	0.80 ± 0.07	0.19 ± 0.03	0.14 ± 0.02	280 ± 59	209 ± 34
	C	2.06 ± 0.22	1.66 ± 0.14	1.15 ± 0.07	1.01 ± 0.08	660 ± 107	491 ± 72
Microencapsulated islets	TNF/γIF	2.39 ± 0.18	0.98 ± 0.10	0.52 ± 0.12	0.48 ± 0.12	537 ± 79	
	C	2.22 ± 0.17	2.53 ± 0.23	2.64 ± 0.23	2.18 ± 0.29	1421 ± 154	
Free islets	TNF/γIF	2.55 ± 0.11	0.79 ± 0.13	0.35 ± 0.05	0.32 ± 0.06	185 ± 35	
	C	2.67 ± 0.21	2.52 ± 0.23	2.31 ± 0.33	1.81 ± 0.33	1001 ± 147	

Insulin was assayed from the culture medium which was replaced at 3-day intervals, day 0 corresponding to secretion from the 3-day run-in period. Residual islet insulin and DNA was measured at the end of the incubation period. C, Paired control free islets or microencapsulated islets not exposed to cytokines; IL-1β, interleukin-1β; TNF, tumour necrosis factor

added to each of four groups of microencapsulated islets and four groups of free islets. Duplicate groups of islets were maintained without IL-1β as controls. The culture media, with or without IL-1β, was changed at 3-day intervals. The media removed was immediately frozen to −70 °C for later insulin assay. At each media change islet morphology was assessed using a stereomicroscope. After 9 days the islets were washed twice in Hanks Balanced Salt Solution (Gibco Ltd.) then three times in normal saline and finally suspended in 250 µl distilled water prior to sonication. One hundred microlitres of islet sonicate was taken and insulin extracted by overnight incubation in acidified alcohol at 4 °C. The remaining 150 µl was assayed for residual DNA content by a fluorimetric assay [11]. Using the same methodology, free and microencapsulated islets were also exposed to a combination of recombinant murine TNFα (final concentration 100 U/ml) (Biogen, Geneva, Switzerland) and recombinant rat IFNγ (gift of Dr. P. van der Meide, The Primate Centre, Tivo, Rijswike, The Netherlands) (final concentration 1000 U/ml).

Insulin was assayed by radioimmunoassay using rat insulin standard (Novo Research Institute, Bagsvaerd, Denmark) and charcoal separation [12].

Statistical analysis

Groups exposed to cytokines were compared to the control group at each time point using Student's *t*-test. Two way analysis of variance was used to compare secretion between groups over the complete 9-day period. The data represent the pooled results of three separate experiments. Static incubation data was similarly compared. All text and figure data are presented as mean ± SEM.

Results

In vivo studies

Islet insulin release in response to glucose challenge following microencapsulation is shown in Figure 1 a. First and second phase insulin release is apparent from both free and microencapsulated islets with similar profiles. There is a slight delay from the microencapsulated islets. Following 6 weeks tissue culture static insulin release from microencapsulated islets had fallen to 35 % (Fig. 1 b). Although a greater reduction was observed from free islets, this did not achieve significance until week 6 ($p < 0.02$).

Size profiles of islet batches prior to transplantation indicated that 70–90 % of islets were of greater than 100 µm diameter and that of these 10–35 % were greater than 200 µm diameter.

Following implantation the blood glucose concentration fell rapidly to hypoglycaemic levels in all five transplanted BB/E rats (Fig. 2) and all animals gained weight (12.2 ± 4.7 g, 2 days post-transplantation). However, relapse occurred between 3 and 14 days post-implantation with progressive hyperglycaemia, polyuria and loss of weight (43.4 ± 16 g, > 10 % body mass for 4/5 animals). Control animals remained hyperglycaemic throughout, lost weight continuously (55.2 ± 16 g at the time of death, > 10 % body mass for 4/5 animals), and all developed severe metabolic decompensation within 6 days.

Microcapsules were mostly found to be lying free within the peritoneal cavity in both groups of animals with only occasional small clumps of capsules seen. On inspection with the naked eye and low power microscopy capsular overgrowth was not apparent and there were no peritoneal adhesions. However, microscopy following fixation and staining revealed fibroconnective and inflammatory tissue covering microcapsules recovered from both groups of animals. Encapsulated islets were degenerating with foamy cytoplasm and pale nuclei even when capsules were intact. Little insulin-positive material was seen in immunostained sections, however, glucagon-positive cells were still present (Fig. 3).

Diabetic streptozotocin Wistar rats given equivalent grafts survived much better although blood sugar readings were more variable (Fig. 2). Weight lost in the 7-day period between chemical induction of diabetes and transplantation was regained within 3 weeks. Bacterial peritonitis was apparent in the animal whose graft failed early, presumably from infection introduced at the time of transplantation. Control animals showed persistent hyperglycaemia and continued to lose weight steadily. Empty microcapsules were removed from control animals at 2 weeks and had minimal or no overgrowth. Islet containing microcapsules recovered 2–6 weeks after implantation showed minimal overgrowth and degenerate islet tissue (Fig. 3).

Collapsed capsules invariably induced a foreign body type reaction.

In vitro studies

Free islets exposed to IL-1β showed a fall in insulin secretion at 3 days with further reduction thereafter (Table 1). A similar effect on microencapsulated islets was seen. The difference in insulin secretion compared to the control

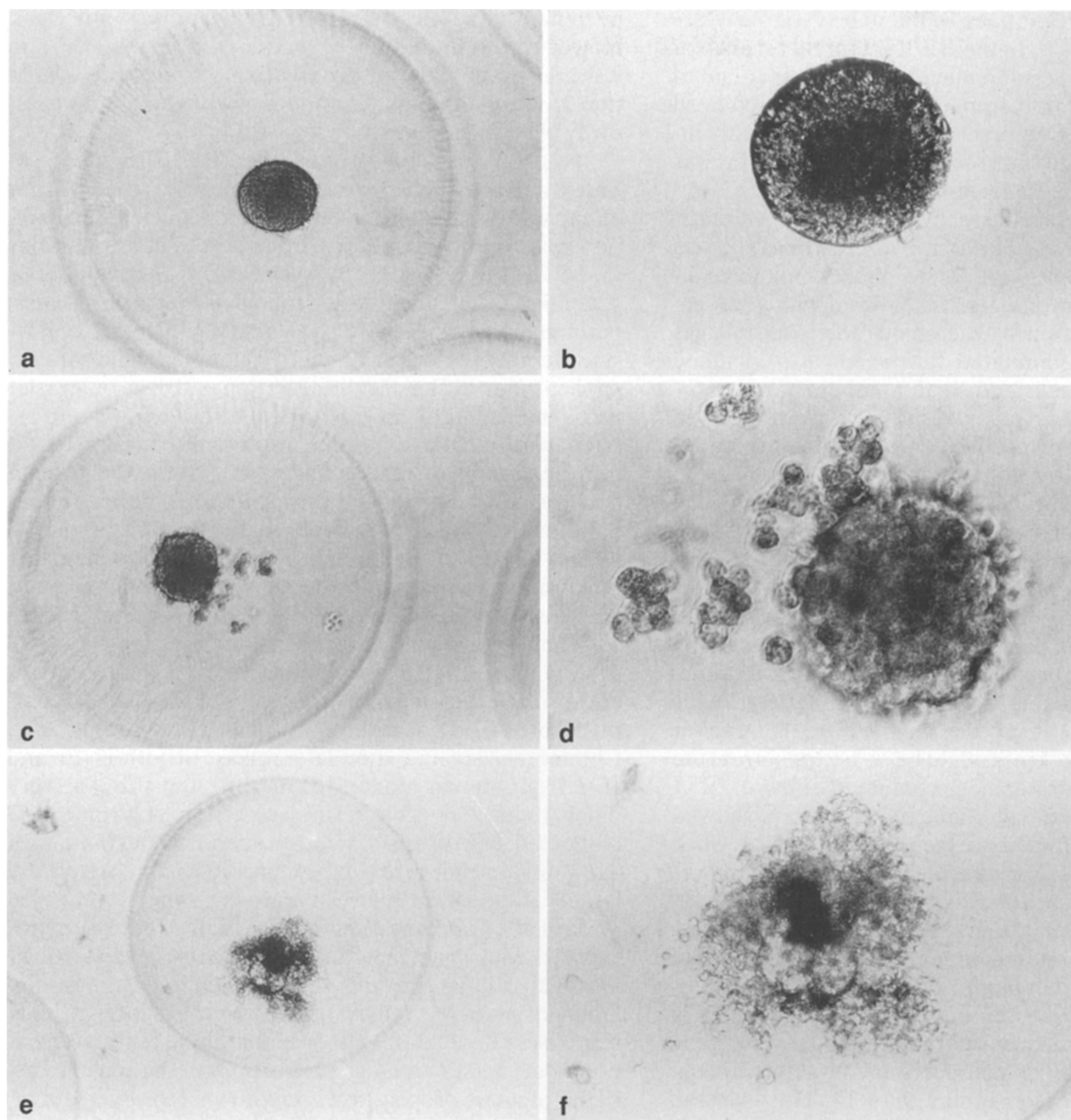

Fig. 4 a–f. In vitro studies: (**a, b**) Control microencapsulated islet after 9 days culture, original magnification ×30 and ×80 respectively; (**c, d**) Microencapsulated islet after 9 days exposure to interleukin-1β, original magnification ×30 and ×80 respectively; (**e, f**) Microencapsulated islet after 9 days exposure to TNFα and IFNγ, original magnification ×30 and ×80 respectively

group for each 3-day period following exposure to IL-1β was significant ($p < 0.01$). There was no significant difference seen in insulin secretion between microencapsulated and free islets.

Total islet insulin content after 9 days exposure to IL-1β was significantly reduced in both free and encapsulated islets compared to the control group ($p < 0.01$ for both). The difference between free and microencapsulated islets was not significant. DNA content of free and microencapsulated islets was also reduced after exposure to IL-1β ($p < 0.05$). Morphological deterioration was observed over the time course of the experiment (Fig. 4). Similar changes were observed following exposure to TNFα and IFNγ in combination (Table 1 and Fig. 4). Morphological damage was usually apparent by 3 days and definitely by 6 days.

Discussion

The functional characteristics of islets microencapsulated using the alginate-polylysine-alginate technique have been reported by others who have shown that in vitro insulin release profiles following secretogogue challenge are similar to those of free islets [9]. We have confirmed these findings here. Studies on insulin release over the longer term in tissue culture have suggested figures ranging from no change over 70 days [13] to a 50 % reduction over 7 days [14]. Our figures lie between these extremes and in terms of insulin release per islet were very favourable at all time points. They also suggested that microencapsulation may prolong islet function under these conditions.

In vivo, long-term survival of microencapsulated islets has been reported in various animal models of diabetes al-

though graft function has been found to be variable in degree and duration [1–3]. In the BB/E rat initial reversal of hyperglycaemia was accompanied by weight gain (a good index of metabolic improvement) beyond that achieved during maintenance treatment with intermittent daily insulin injections prior to transplantation. However, this was poorly sustained, relapse occurring in all implanted animals within 14 days. Freshly recovered microcapsules, although apparently free of cellular overgrowth as seen on low power microscopy, were found on examination, following fixation and staining, to be covered with a foreign body type tissue reaction in which giant cells, macrophages and fibroblasts predominated but which also included other mononuclear cells and small numbers of polymorphonuclear leucocytes. Capsular overgrowth was seen in all animals studied irrespective of whether the microcapsules contained islets or not and was particularly marked around the small number of collapsed capsules from which the islets had disappeared. Upon immunostaining islets within intact capsules also appeared to have suffered damage suggesting preferential Beta-cell loss. This implies penetration of the capsule by factors cytotoxic to islets released by cells in the overgrowth. It has been shown by others that such microcapsules are impermeable to molecules larger than albumin [15] and in particular to cytotoxic antibodies [16]. It seemed possible, however, that smaller molecules such as cytokines might be able to penetrate the capsular membrane and damage contained islets.

Grafts in streptozotocin-diabetic Wistar rats showed significantly longer function except in one animal which developed peritonitis soon after transplantation. Numbers of islets transplanted are usually quoted in microencapsulation experiments but this is a poor index of islet mass, which also depends on individual islet size. We recorded profiles of islet diameter but have nothing to compare this data with from other groups. Viability may also vary. Function following microencapsulation has been discussed above and in our hands compares favourably with the limited data available from others [9, 13, 14]. However, the shorter duration of graft function in our streptozotocin diabetic rats compared to that found by some other authors using non-autoimmune models [1, 2, 14] could still be the result of lesser amounts of transplanted islet tissue. By displaying the progress of individuals rather than grouped animals variability within a given individual was highlighted. One potential pitfall, easily mistaken for prolonged graft function, is the recovery of host pancreas Beta-cell function following transplantation [17]. However, the discrepancy between graft function in BB/E and streptozotocin diabetic Wistar rats was still clearly demonstrated.

Empty microcapsules recovered from control streptozotocin diabetic Wistar rats after 2 weeks showed little or no tissue overgrowth in complete contrast to the response in BB/E rats. Microcapsules from transplanted Wistar rats also showed minimal overgrowth. These had been recovered later than the other microcapsules, 2–6 weeks after implantation, which probably explains why they were sometimes not quite as clear of adherent tissue, but still much less overgrowth was found than in the BB/E model over a shorter time course. Some eosinophilic material, presumed to be islet debris, was found within the microcapsules. The cause of islet death was not clear. However, the duration of function and gradual decline was consistent with our observations on long-term function in vitro, although function in vitro would not necessarily be a guide to function in vivo.

It has been demonstrated that IL-1β (mol wt. = 17,500), TNFα and IFNγ (both with mol wt. < 25,000) are all capable of damaging islet tissue and reducing islet function in vitro [6, 7]. In particular, under defined conditions IL-1β destroys Beta cells specifically, although more generalised islet damage then follows [18]. The in vitro experiments described here show that the alginate-polylysine membrane does not exclude cytokines, which are capable of suppressing insulin secretion and destroying islet tissue, indicated by morphological disintegration and reduction in both islet DNA and insulin content. Biomedical polymers activate macrophages as part of the foreign body response and have been shown to stimulate the release of IL-1 [4]. It is possible that activation of macrophages as part of the foreign body response to alginate–polylysine microcapsules with subsequent penetration of the capsule by IL-1β (and/or other cytokines) could be responsible for the graft failure seen in these animals.

Alginate, the supporting structure for many microencapsulation systems, is a linear polysaccharide composed of 1–4 β-D-mannuronic acid and/or guluronic acid. Commercial alginate stimulates release of both TNFα and IL-1 from human monocytes in vitro, but this was very much reduced in gels formulated from guluronic acid alone [19]. Microcapsules constructed from alginate with a low mannuronic acid (high guluronic acid) content were largely free of cellular overgrowth when recovered 21 days after implantation in Lewis rats, whereas microcapsules with a high mannuronic acid content were associated with cellular overgrowth. It was suggested that stimulation of cytokine release might recruit a foreign body response. This data was not available at the time of our experiments and the relative proportions of the constituents of our alginate preparation is unknown. However, our alginate-polylysine-alginate microcapsules produced no or minimal foreign body reaction in Wistar Furth rats and only elicited a significant response in BB/E rats. Thus, tolerance to the microcapsules is, at least in part, dependent on the host strain. In addition, we have found that severity of capsular overgrowth also varies on an individual basis within a given strain of animals [20].

Sun and co-workers have reported successful engraftment of microencapsulated islets in spontaneously diabetic BB/W rats although cellular overgrowth was seen in a proportion of recovered capsules and some recipients required regrafting [3]. In contrast, Mazaheri et al. have recently reported failure of microencapsulated allogeneic islet grafts at around 14 days in diabetic BB/W recipients, and that their capsules were surrounded by a dense pericapsular infiltrate, in agreement with the finding reported here [21]. There may have been differences in the microcapsules, but this disparity could also be accounted for by variable tolerance to capsules occurring in different groups of animals.

A somewhat different situation apparently exists in the spontaneously diabetic NOD mouse. Here, a destructive

cellular reaction has been observed to microencapsulated islet xenografts but not to empty microcapsules. Further, this response could be abolished and graft function prolonged by in vivo treatment with anti L3T4 monoclonal antibody [22]. In our experiments there was no discernible difference in the reaction to empty, as compared to islet-containing, microcapsules.

Immunoisolation using an alternative membrane in the form of a hollow fibre did protect islets within against IL-1β over a 2-day incubation period [23]. This result is surprising as the membrane had a molecular weight cut-off of about 50,000 and would be expected to allow free passage to molecules smaller than this. Other factors, including molecular geometry, chemical and physical properties and binding of proteins may also affect permeability. Although obviously technically difficult, it is not inconceivable that modifications to the microencapsulation membrane might be made to try and achieve similar protection.

An additional explanation for the rapid decline in function and islet damage within microcapsules surrounded by reactive cells could be starvation. The encompassing tissue might create not only a physical barrier to the passage of islet nutrients (and insulin) but would also be competing consumers in their own right.

In conclusion, early graft failure of microencapsulated islet grafts in established spontaneously diabetic BB/E rats was accompanied by a marked foreign body type reaction and islet damage which may preferentially affect the Beta cell. Cytokines have been shown to cross the microcapsule membrane in vitro and may be responsible for the islet damage and graft failure observed within intact capsules in engrafted animals. There appears to be some variation in response to both microcapsules and microencapsulated islets depending upon the animal model employed. For this reason it may be difficult to extrapolate the results of these experiments with microcapsules to man.

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Dr. D. R. Cole
Department of Medicine II
Level D, South Block
Southampton General Hospital
Southampton SO9 4XY
UK