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Transplantation of microcapsules (a potential bio-artificial organ): biocompatibility and host reaction

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Tolerance to alginate–polylysine–alginate microcapsules implanted into the peritoneal cavity was compared in the Wistar Furth rat and the BB/E (Wistar-derived) spontaneously diabetic rat. A marked foreign-body type reaction was observed in the BB/E rat in both diabetic and non-diabetic animals. In contrast, little or no reaction was observed in the Wistar Furth rat. Implantation under the kidney capsule, an immunologically privileged site, did not protect the microcapsules. Blocking the surface charge of the microcapsule by coating with tolylene diisocyanate also failed to modify the reaction. Coating with a water-insoluble lacquer (Eudragit RL) resulted in dense capsule overgrowth. Thus tolerance to alginate–polylysine–alginate microcapsules appears to be dependent upon the recipient animal strain and this may explain some of the discrepancies in function observed in different animal models when this system has been used to encapsulate pancreatic islets for a bioartificial pancreas. The tissue reaction does not seem to be affected by clinical diabetic status although abnormal immunological responses in animals with a tendency to spontaneous diabetes could be important. Attempts to reduce the reaction to the capsules in the BB/E rat strain by modifying the membrane were unsuccessful.

1. Introduction

Microencapsulation seeks to protect grafted cells and tissues from rejection, the semipermeable membrane allowing exchange of nutrients and products whilst protecting against immunocytes and cytotoxic factors, including antibodies. As hybrids of animal tissue and a synthetic membrane, they have been termed "bioartificial organs". One such bioartificial organ, the bioartificial pancreas, has been of particular interest [1] as diabetes mellitus is a common condition and organ replacement therapy in the form of pancreatic islet transplantation has recently proved successful in man [2]. Pancreatic islets, by virtue of their small size and capacity for independent survival, are ideally suited to microencapsulation and use as a bioartificial pancreas. Systemic immunosuppression of the patient could be avoided and there is the possibility of xenotransplantation (e.g. islets from porcine donor pancreas). Implantation of microencapsulated islets is technically straightforward and offers the hope of safe treatment early in the disease prior to the development of the complications of diabetes.

Several microencapsulation systems have been proposed for use as a bioartificial pancreas. The original technique used in this context consisted of islets enclosed within alginate beads on which layered poly-1-lysine followed by polyethylenimine [3]. Although function was demonstrated in the short term, this was not sustained and recovered microcapsules were found to be overwhelmed by an extensive inflammatory response. This became much less of a problem when an outer coating of alginate was substituted for polyethylenimine. However, function appears to be variable and an inflammatory reaction still sometimes evident [4–9]. Biocompatibility remains a major obstacle in the development of many bioartificial systems, including other proposed bioartificial pancreata [10, 11].

In the present work, we have directly compared the biocompatibility of microcapsules implanted into two animal models of insulin-dependent diabetes. The Wistar Furth rat can be made diabetic by injection of streptozotocin which selectively damages the insulin-producing beta cell and can, therefore, be categorized as a chemically-induced model [12]. The spontaneously diabetic BB rat was Wistar-derived and has a strong tendency to spontaneous insulin-dependent diabetes mellitus which is autoimmune in origin and appears to mimic the human clinical condition [12]. Histology of recovered microcapsules following implantation into the peritoneal cavities of both models was compared. Tolerance to both pancreatic islets [13] and microcapsules [14] may depend upon the site of implantation. Response to microcapsules im-

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planted into the renal subcapsular space, a possible alternative to the peritoneal cavity for graft implantation, was evaluated to see if additional protection was afforded at this site.

Modifications to the alginate–polylysine–alginate (APA) microcapsules have been described which may affect biocompatibility [15, 16]. Different coatings appear to change the surface characteristics of microcapsules, isocyanate blocking the surface charge, polyacrylate creating a hydrophobic lacquer. However, no histological comparison has been reported. APA microcapsules were therefore compared with alginate–polylysine–isocynate and alginate-polyacrylate microcapsules. Depth of overgrowth and relative proportions of clear, overgrown and collapsed capsules were recorded.

2. Materials and methods

2.1. Microencapsulation

Microcapsules were produced precisely according to the method of Sun and colleagues [3, 17]. A 1.5% wt/vol sterile stock solution of sodium alginate (Kelco Gel LV, Kelco, New Jersey, USA) in normal saline was serially filtered through 0.1 mm, 0.5 and 0.22 μm filters. The alginate solution was drawn up into a plastic syringe which was attached to an 18 gauge stainless steel needle, in turn surrounded by a purpose-built machined air jacket. The syringe was driven by a pump and both the rate of air flow through the air jacket and the syringe pump speed were adjusted to optimize microcapsule parameters (perfectly spherical 600 μm diameter microcapsules). The droplets produced were collected into a solution of 100 mmol/l calcium chloride, the surface of which was 4 cm from the needle tip. Upon contact, the droplets form solid beads which collect under gravity at the bottom of the tube. Following three washes in normal saline, the beads were treated with 0.1% CHES (2-*N*-(cyclohexylamino)ethone-sulphonic acid) (Sigma Chemical Company, St Louis, USA) buffer pH 8.4 for 3 min whilst being gently rotated. A further wash in normal saline was followed by coating with poly-1-lysine, molecular weight 17 000 (Sigma Chemical Company, St Louis, USA) as a 0.05% solution in normal saline for 10 min followed by a wash in CHES buffer for 3 min. A wash in normal saline was followed by coating with 0.15% sodium alginate in normal saline for 4 min. Alginate and polylysine bind to produce a stable alginate–polylysine–alginate membrane. After two washes in normal saline, the alginate core was resolubilized by suspension in 55 mM sodium citrate buffer pH 7.4 for 6 min. Finally, the microcapsules were washed three times in normal saline.

2.2. Animal models

The Edinburgh BB rat colony (subsequently designated BB/E) was established in 1982, the nucleus of which was kindly donated by Dr Pierre Thibert from the colony maintained at the Animal Resources Division of Canada, Ottawa (designated BB). The BB/E colony consists of two sublines of animals created by selective outbreeding. The diabetes-prone (DP) subline has a diabetes incidence of approximately 60% with a mean age of onset of 96 days. The diabetes resistant (DR) subline has an incidence of < 1% at 120 days of age. Animals with diabetes are maintained with daily injections of highly purified monocomponent insulin (Ultralente; Novo Nordisk, Copenhagen, Denmark). Wistar Furth rats were made diabetic 1–2 weeks prior to study by intravenous streptozotocin, 45 mg/kg body weight (Sigma Chemical Company) 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 used in the studies were adults of between 180 and 270 days of age.

2.3. Tolerance to APA microcapsules in BB/E and Wistar rats

Empty microcapsules were produced in batches of approximately 3000. Around 1000 such microcapsules were placed into the peritoneal cavity of the following groups of rats: adult Wistar Furth, streptozotocin-induced diabetic Wistar, BB/E (DR) non-diabetic, BB/E (DP) non-diabetic and BB/E (DP) established diabetic. A 10 ml syringe containing microcapsules suspended in approximately 4 ml sterile saline was attached to a large-bore butterfly cannulation system. Care was taken to ensure the capsules were well mixed to avoid blocking the tube and possibly damaging them. Under halothane general anaesthesia, the butterfly needle (14 gauge) was inserted into the midline of the abdomen two-thirds of the distance from the xiphersternum to pubic synthesis and the capsules slowly injected, free flow indicating clear passage into the peritoneal cavity.

After 2 weeks the capsules were removed by laparotomy and lavage with physiological saline. In some animals this was repeated after a further 2–4 weeks. General appearance was noted under low-power light microscopy and the recovered material prepared for histology as described below.

2.4. Comparison of subrenal capsular and intraperitoneal sites

Groups of 50–100 microcapsules were placed under the kidney capsule of recipient BB/E established diabetic rats using a loin incision under halothane general anaesthesia. The kidney was exposed by gentle traction and a small incision through the kidney capsule (approximately 2 mm long) was made at the upper pole of the lateral border of the kidney using a scalpel blade. The capsule was then lifted off the kidney surface by inserting a round-ended mouth gavage tube. The microcapsules were drawn up into a 17 gauge cannula via an improvised aspirator (rubber tubing attached to a mouth piece) and transferred about 20 at a time to the space created under the kidney capsule. When the pocket was full the same procedure was repeated at the other pole. The kidney was kept moist with saline throughout and, after about 100 microcapsules had been implanted, the

wound was closed in layers with 4/0 silk and the skin with clips.

After 2 weeks the kidneys were removed and fixed in formalin prior to paraffin embedding and sectioning as described below.

2.5. Comparison of modified microcapsules

Microcapsules with outer coatings of alginate (the standard procedure), an acrylic/methacrylic hydrophobic ester copolymer (Eudragit RL) [15] or isocyanate [16] were implanted into the peritoneal cavity of mature established diabetic BB/E rats by the butterfly cannulation system described above. After 2 weeks the animals were sacrificed and capsules recovered for histology.

Eudragit RL coating: a 5% emulsion was made by adding Eudragit RL 100 resin (an acrylic acid/methacrylic ester copolymer containing a low content of quaternary ammonium groups, molecular weight 150 000) (Rohm Pharma GmbH, Darmstadt, Germany) to vigorously boiling glass-distilled water. Care was required to prevent the mixture from foaming. From this stock solution a 0.5% (wt/vol) emulsion was prepared with isotonic *tris*-calcium chloride buffer pH 7.4 (135 mmol/l sodium chloride; 10 mmol/l calcium chloride: 1 mmol/l Tris). Alginate beads were made according to the standard protocol. These were then washed in *tris*-calcium chloride buffer and coated with Eudragit RL by gently shaking the beads for 30 min in the 0.5% emulsion. The capsules were again washed in buffer and then in normal saline prior to implantation.

Isocynanate coating: a 0.2% solution of isocynanate was made as follows: 0.5 ml 10% aqueous Brij 58 highly purified detergent ("Surfact-amp", Pierce Warriner Ltd, Chester, UK) was added to 0.6 ml 2 × phosphate-buffered saline (sodium chloride 16 $g\,l^{-1}$, potassium chloride 0.4 $g\,l^{-1}$, disodium hydrogen phosphate 2.3 g l, potassium hydrogen phosphate 0.4 $g\,l^{-1}$; dissolved in 1 l distilled water, pH 7.2), 0.026 g tolylene 2,4,-diisocynate was added and sonicated on ice until mixed (about 10 min) and then diluted to 13 ml with single-strength phosphate-buffered saline. Polylysine-coated alginate beads were made according to the standard protocol and, after washing in CHES buffer and twice in normal saline, were suspended in 10 ml 0.2% isocyanate solution for 1 min with constant gentle mixing and then washed a further four times in normal saline prior to implantation.

2.6. Preparation of specimens for histological examination

Tissue was fixed in formalin at room temperature overnight. The capsules were then aspirated using a large-bore plastic pipette and collected in small bags fashioned from 200 μm nylon mesh (Henry Simon Ltd, Stockport, UK) prior to automated paraffin embedding by vacuum infiltration ("tissuetek" system, Miles Laboratories, Slough, UK).

Sections 4 μm long were cut from the paraffin blocks and stained with Harris' haematoxylin and eosin. Recovered microcapsules from each animal were scored as clear, overgrown or collapsed. For the comparison of capsule types, the depth of overgrowth was determined using an image analyser (VIDS II, Analytical Measuring Systems Ltd, Saffron Walden, UK). Thickness was determined at four points at right angles around the capsule to give the average depth for each capsule type. These data were presented as mean ± standard deviation and significance assessed using Student's *t*-test.

3. Results

3.1. Tolerance to APA microcapsules in BB/E and Wistar rats

At laparotomy, microcapsules were observed to be free within the peritoneal cavity with only the occasional clump. There was no evidence of fibrosis or adhesions. Between 50% and 70% of the microcapsules implanted (by volume) were recovered. Under low-power microscopy (prior to fixation and staining) the microcapsules from all groups of animals appeared to have been unaffected by implantation. However, histological examination demonstrated that in all groups of recipients the small numbers of damaged microcapsules had elicited a foreign-body response. Intact microcapsules had evoked a minimal response, if any, in normal Wistar and streptozotocin diabetic Wistar animals, but in the BB/E rats a marked foreign-body response was associated with intact microcapsules in both DP and DR sublines. Similar changes were observed for microcapsules obtained 4–6 weeks after implantation, with more extensive reaction, although this was not quantified. Diabetes *per se* did not appear to affect the response (Table I). Representative sections are shown in Fig. 1.

Additionally, mesentery and mesenteric, para-aortic, cervical and inguinal lymph nodes and the spleen were recovered from the five BB/E established diabetic rats. No changes were seen in response to the intraperitoneal implantation of microcapsules in these tissues.

3.2. Comparison of subrenal capsular and intraperitoneal sites

Microcapsules implanted under the kidney capsule recruited fibroconnective tissue together with inflammatory cells (Fig. 1). Although the response appeared

TABLE I Comparison of tolerance to microcapsules in Wistar and BB/E rats (means ± S.D.)

Recipient	*N*	Capsules with overgrowth (%)	Capsules collapsed (%)
Wistar	10	< 1 ± 1	12 ± 7
Wistar-STZ (diabetic)	6	2 ± 2	3 ± 3
BB/E (DR)	5	41 ± 26	8 ± 5
BB/E (DP)	8	75 ± 19	19 ± 18
BB/E (DP) (diabetic)	10	51 ± 37	15 ± 15

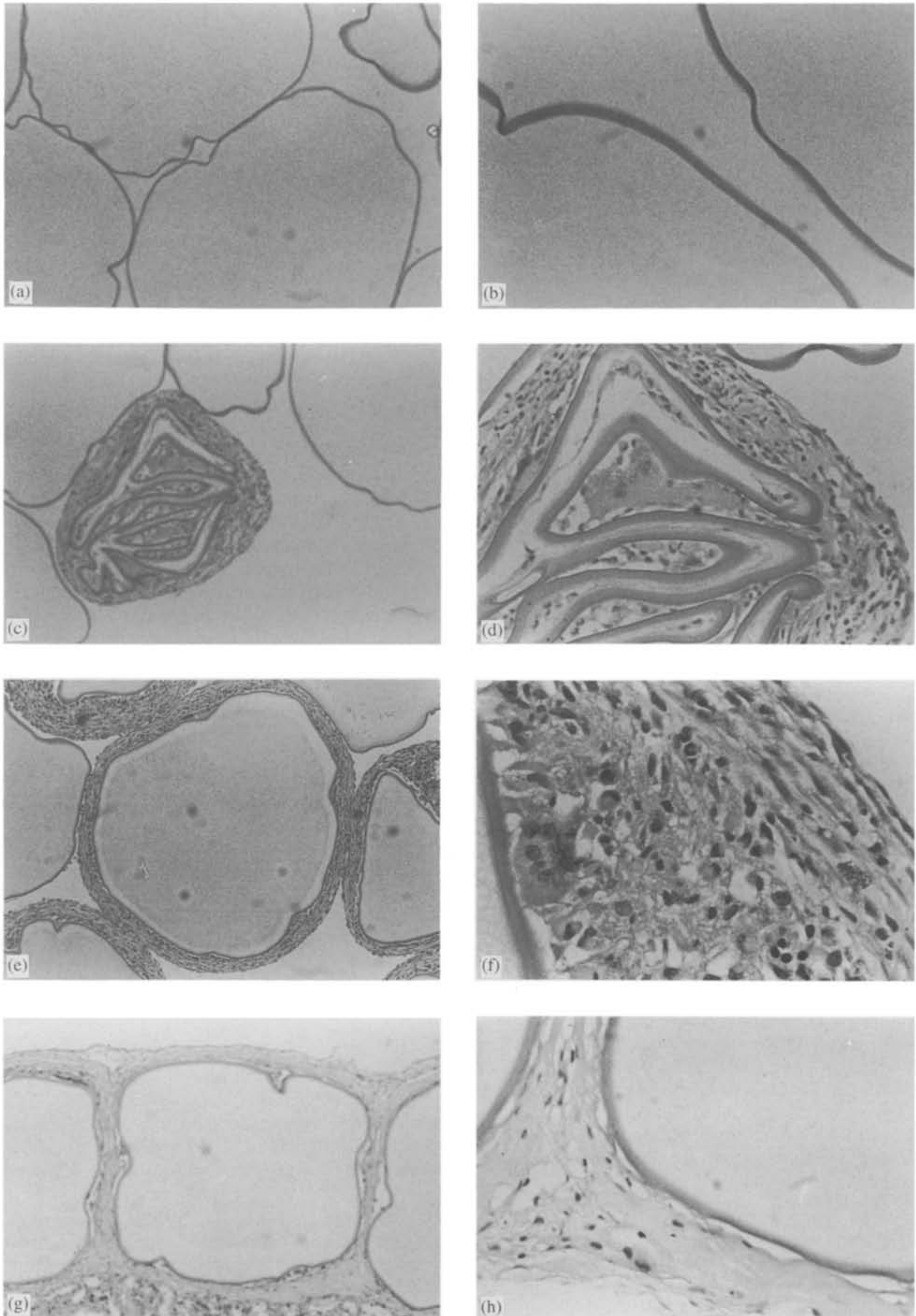
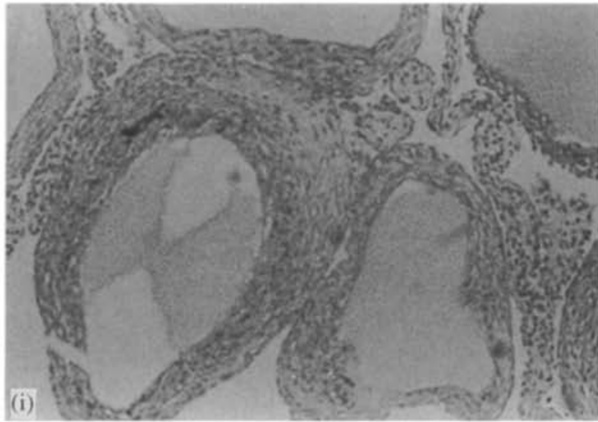


Figure 1 Photomicrographs of recovered microcapsules. (a, b) APA into Wistar, intraperitoneal (original magnification × 30 and × 80, respectively); no evidence of tissue reaction against intact microcapsules. (c, d) APA into Wistar, intraperitoneal, showing reaction to collapsed microcapsule (original magnification × 30 and × 80, respectively). (e, f) APA into BB/E rat (original magnification × 30 and × 112.5, respectively). Marked tissue reaction to intact microcapsules. (g, h) APA into BB/E under kidney capsule (original magnification × 30 and × 80, respectively). Marked reaction. (i, j) A-Polyacrylate into BB/E intraperitoneal (original magnification × 80 and × 112.5, respectively). Severe tissue reaction was observed. (Results from API into BB/E microcapsules were similar to those of APA microcapsules).



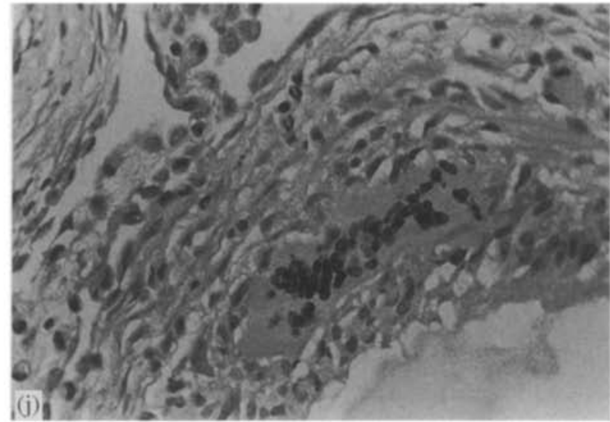


Figure 1 (Continued)

TABLE II Tolerance to different microcapsules in BB/E (DP) diabetic rats ($n = 10$ for all groups, means $\pm$ S.D.)

Microcapsule type	Capsules with overgrowth (%)	Capsules collapsed (%)	Depth of overgrowth (μm)
Alginate–polylysine–algine	51 ± 37	15 ± 15	48.5 ± 13.8
Alginate–polylysine–isocyanate	51 ± 18	27 ± 17	48.0 ± 12.8
Alginate–polyacrylate	61.8 ± 17	23 ± 14	73.8 ± 9.5

Depth of overgrowth: APA versus API n. s.; APA versus A-Polyacrylate $p < 0.05$; API versus A-Polyacrylate $p < 0.05$.

less than in the peritoneal cavity, this may have been because of compression of tissue within a limited space. There was some distortion of microcapsule architecture apparent before (under low power microscopy) as well as after fixation.

3.3. Comparison of modified microcapsules

Microcapsules again appeared to be free at laparotomy and without adherent tissue on low-power microscopy. Following histological preparation, a foreign body response was apparent in BB/E rats and the response to different coatings with relative proportions of clear, overgrowth and collapsed microcapsules and depth of adherent tissue is shown in Table II. Collapsed microcapsules were invariably covered with adherent tissue but were not counted twice. There was significantly greater overgrowth around Eudragit-coated microcapsules compared to either isocyanate or alginate-coated microcapsules ($p < 0.01$). There was no significant difference between isocyanate- and alginate-coated microcapsules. In Wistar rats, all types of microcapsules were mostly clear of overgrowth.

4. Discussion

The cellular overgrowth of fibroblasts, macrophages, giant cells, some polymorphonuclear cells and collagen deposition found covering many of the microcapsules after implantation in BB/E rats is typical of a foreign-body type reaction. Others have also found cellular overgrowth using these capsules. O'shea *et al.* described capsules recovered from five Wistar rats as having some host cells attached to the outer surface [17]. This group felt that the following characteristics were essential for biocompatibility: construction of capsules using a highly purified low-viscosity alginate, spherical shape, increased thickness of polylysine coating and an outer coating of alginate (originally polyethylenimine had been used for the outer coating with severe capsular overgrowth). In a later study, unfixed and unstained microcapsules recovered from the peritoneal cavity of transplanted animals were shown to substantiate claims of biocompatibility but without supportive histology [18]. In our own studies, unprepared microcapsules always appeared free of adherent tissue under low-power microscopy. Occasionally a small degree of clumping was seen. It was only after embedding and staining that overgrowth became apparent.

In a study of microencapsulated rat islets implanted into streptozotocin diabetic BALB/c mice, O'Shea and Sun [4] reported varying degrees of overgrowth up to ten cell layers in thickness with fibroblasts, macrophages, neutrophils and collagen in 12 of 20 animals. In one study using the BB/W rat, overgrowth was found over about 30% of capsules [5]. Despite this, graft function of several months was observed, although some animals required "top up" transplants. In contrast, Mazeheri *et al.* [7] described significant overgrowth in BB/W rats and observed a close correlation between extent of overgrowth and graft failure, which occurred in most animals within a few days of transplantation. We have also reported adverse effects of overgrowth on microencapsulated islet grafts in BB/E rats and suggested a mechanism whereby cytokines from the inflammatory cells might directly

damage islet tissue [19]. This prompted our studies on modified microcapsules to see if the reaction could be blocked or reduced.

It has been suggested that success or otherwise of microencapsulated islet transplants is a consequence of materials used and/or preparation methods in different laboratories. Both changing the source of poly-1-lysine [20] and alginate [21] have been shown to have effects on tolerance to microcapsules. Our findings, that biocompatibility of APA microcapsules is good in Wistar rats but poor in BB/E rats from the same production run suggests that response cannot be ascribed to materials and methods alone. The importance of structural integrity is suggested by the increased reaction observed against broken capsules.

Ricker *et al.* [20] reported an intensified reaction around microencapsulated porcine islets implanted into spontaneously diabetic NOD mice as compared to empty control microcapsules. No difference between empty and islet-containing microcapsules has been reported in other models. Further studies in the NOD mouse supported this finding which was preventable by prior treatment of the recipient with systemic anti-CD4 monoclonal antibody, implicating CD4 + lymphocytes in the immune response [6]. Cyclosporin and dexamethasone also reduced reaction to microcapsules in the BB/W rat and prolonged microencapsulated islet graft function [7]. Degree of overgrowth again correlated with graft failure. However, because the rationale behind the development of bioartificial organs is to avoid the need for immunosuppression, this approach to overcoming the problems of biocompatibility appears self-defeating.

It is likely that surface characteristics of the microcapsule membrane are important in determining biocompatibility [17]. We compared three different outer coatings which have been described as having quite different effects on the membrane. The alginate coating is hydrophilic, Eudragit RL is hydrophobic and isocyanate blocks surface charge. Eudragit RL effected a vigorous response to the microcapsules in the BB/E rats, despite theoretical advantages [15]. Although a previous report based on macroscopic studies had implied improved tolerance [16], no advantage of isocyanate over alginate was observed here. A variety of other materials have been used in encapsulation systems but foreign-body type reactions continue to limit their application *in vivo*. The development of *in vitro* assays to predict *in vivo* tolerance would obviously be of advantage.

In conclusion, tolerance to alginate-polylysine microcapsules is dependent not only on the construction of the microcapsule itself, but also upon the recipient animal and the substrain to which it belongs. Biocompatibility is an essential prerequisite for function of bioartificial organs, but the variable tolerance of these animals to microcapsules questions their validity as models predictive of tolerance and function in man.

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