

Deletion of the tissue response against alginate-pll capsules by temporary release of co-encapsulated steroids

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Abstract

Transplantation of encapsulated living cells is a promising approach for the treatment of a wide variety of diseases. Large-scale application of the technique, however, is hampered by inflammatory responses against the capsules. In the present study, we investigate whether tissue responses against alginate-PLL-alginate capsules can be modulated by co-encapsulation and temporary release of immunomodulating factors such as dexamethasone. Such an approach may be mandatory in order to increase the function and survival of encapsulated tissue since it has been shown that the tissue response can be caused by many, insurmountable factors. In an *in vitro* assay, we demonstrated an antiproliferative effect of dexamethasone-containing capsules on L929-mouse-fibroblasts. Subsequently, capsules prepared of purified alginate with or without solved dexamethasone were implanted in the peritoneal cavity of rats and retrieved one month later for histological evaluation. Most of the capsules without dexamethasone proved to be overgrown and adherent to the abdominal organs whereas with co-encapsulated dexamethasone the majority of the capsules were found freely floating in the peritoneal cavity without overgrowth. We conclude that co-encapsulation of dexamethasone has a profound effect on fibroblasts and macrophages adherence to immunisolating capsules.

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1. Introduction

Transplantation of cells for treatment of human disorders such as hormone deficiencies is only applied on limited scale as a consequence of the necessity to use life-long immunosuppression for preventing rejection of the graft. To overcome the obstacle of application of immunosuppression, many investigators have focussed on the design of techniques to encapsulate hormone-

secreting cells in semipermeable membranes to protect donor-cells against antibodies and cytotoxic cells of the host immune system [1,2].

A commonly used procedure for immunoprotection is microencapsulation of tissues in alginatepoly-L-lysine (PLL) based capsules as originally described by Lim and Sun [2]. During recent years, important advances have been made with this technology. The first allotransplantations in humans with encapsulated parathyroid cells [3] and islets [4] have been successfully performed [4]. Although this illustrated the principle applicability of the alginate-encapsulation technique, a fundamental barrier has to be overcome since graft

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survival varies considerably from several days to months [5–10].

The variation in success rate is usually attributed to differences in the chemical composition [11–13] and, consequently, differences in biocompatibility of the applied capsules. A major factor causing bioincompatibility is insufficient covering of positively charged PLL at the capsules surface [6,13,14] which has been shown to induce foreign body reactions by attracting macrophages and fibroblasts [13,14].

As the tissue response predictably interferes with survival of encapsulated cells [11–13], efforts should be made to suppress activities or recruitment of inflammatory cells. Recently, it has been shown that the tissue response is initiated immediately after implantation and consists of more than one type of reaction [15]. The most commonly known response is the foreign body reaction against the capsule's materials but, recently, it has been shown that equally important is the non-specific immune activation caused by the surgical trauma of implantation. The latter response is difficult to prevent and is inevitably associated with fibrotic overgrowth of a significant portion of the capsules.

In the present study we have investigated whether the tissue response against capsules can be reduced by release of immunomodulating agents such as dexamethasone in the immediate vicinity of the graft. This local release near the graft site instead of systemical administration can prevent the undesired side effects associated with systemical release of immunosuppressive agents. These efforts may be temporary rather than permanent since most inflammatory cells have disappeared after 2 weeks of implantation [15].

In the present study we have compared the tissue responses against empty alginate-PLL-alginate capsules with those against dexamethasone-containing capsules after intraperitoneal implantation in rats. Prior to the experiments we have studied *in vitro* the release and the biological activity of encapsulated dexamethasone for prolonged periods of time.

2. Materials and methods

2.1. Design of the study

Before implantation, we have studied, *in vitro*, the release and biological activity of the co-encapsulated dexamethasone. Dexamethasone release was studied over 14 days after co-encapsulation to study the profile of release of dexamethasone. In order to confirm that the released dexamethasone maintains its biological activity, L929-mouse-fibroblasts were encapsulated in capsules with and without dexamethasone. The proliferative activity and viability of the L929-mouse-fibroblasts was assessed at several days after encapsulation.

Alginate-PLL-alginate microcapsules with dexamethasone (i.e. dexamethasone-capsules) and without dexamethasone (i.e. conventional capsules) were implanted in the peritoneal cavity, that is the usual transplantation site for a capsule graft, of Wistar-rats. The capsules were inspected before and after implantation in order to confirm that the majority of the capsules was intact. The capsules were retrieved at four weeks after implantation to study the degree of overgrowth, i.e. a measure for the capsules biocompatibility.

2.2. Cell cultures

Mouse fibroblasts L929 (ACC2, DSMZ, Braunschweig, Germany) were cultured as described elsewhere [16]. The applied cells were never used after passage 12. Medium was changed 2 times a week.

2.3. Capsule production

Purified alginates [5,17] were dissolved at 40 °C in KRH with an appropriate osmolarity. Before application the solutions were sterilized by 0.2 µm filtration.

The 3% alginate solution was converted into droplets using an air-driven droplet-generator [18]. Alginate-poly-L-lysine-alginate encapsulation was performed as described elsewhere [11]. The alginate droplets were transformed to alginate beads by gelling in a 100 mM CaCl₂ solution (containing 10 mM HEPES, 2 mM KCl) for at least 10 min. After washing with KRH containing 2.5 mM CaCl₂ for 1 min a poly-L-lysine (PLL) (PLL-HCL, 22 kDa, Sigma-Aldrich, Germany) membrane was formed by suspending the beads in a 0.1% PLL solution for 10 min. Subsequently, the coated beads were washed three times in Ca²⁺-free KRH containing 135 mM NaCl for 3 min. Finally, the capsules were suspended for another 5 min in a 0.3% solution of alginate.

Dexamethasone capsules were produced in an identical fashion. The only difference was that the alginate solution was mixed with dexamethasone in a ratio of 4 mg/1.5 ml.

L929-mouse-fibroblasts containing capsules were produced by gently mixing 2 × 10⁶ cell per ml of 3% alginate-(conventional capsules) or dexamethasone-alginate-(dexamethasone capsules) solution.

After the procedure the capsules had a diameter of 650 ± 50 µm.

2.4. Biological activity of dexamethasone

The biological activity of dexamethasone was measured by assessing the proliferation rate of encapsulated L929-fibroblasts in conventional or dexamethasone capsules since dexamethasone has been shown to have a clear-cut and profound effect on the proliferation of L929-fibroblasts [19,20]. This proliferation was

measured on day 0, 1, 3, 7, 14, 21, 28, 35 and 42 using the MTS /PMS-assay (Promega, Mannheim, Germany) as previously described [16]. Briefly, 20 fibroblast-containing capsules were withdrawn from the culture-dish and brought into a 96-well tissue culture plate with 100 μ l capsule-medium-volume/well and 20 μ l MTS/PMS-solution. Incubation was performed for 4 hours at 37 °C. The amount of reduced formazan was assessed by reading the optical densities at 492 nm with 620 nm as a reference against blanks using culture media without encapsulated cells.

To test the biological activity of steroids that have diffused to the outside of the capsule we performed cocultures of 1×10^5 non-encapsulated fibroblasts with 20 alginate-PLL capsules with or without dexamethasone. Proliferation rates were assessed by a MTS/PMS-assay as described above.

Each test was repeated at least four times in order to exclude variations.

2.5. Cell-survival assay

The survival of encapsulated cells was measured with the Life-Dead-Assay (MobiTec, Göttingen, Germany) as described elsewhere [16]. Briefly, samples of 20 capsules were taken from the culture-dish on day 0, 1, 3, 7, 14, 28, and 42. After PBS-washing the ethidium-calcein mixture was added and incubated for 30 min in a 37 °C-incubator. After another washing with PBS the capsules were examined under a fluorescent-microscope (Nikon, Düsseldorf, Germany) for quantifying living (green stained) and dead (red stained) cells.

2.6. Graft recipients and surgical procedures

Male Wistar/R rats served as recipients of microcapsules and were obtained from the Central Animal Laboratory of Rostock, Germany. Their body weights ranged from 300 to 350 g.

Capsules were injected into the peritoneal cavity with a 16 G cannula via a small incision (3 mm) in the linea alba. The abdomen was closed with a two-layer suture. The implanted volume was always 0.5 ml as assessed in a syringe with appropriate measure.

The microcapsules were retrieved four weeks after implantation by peritoneal lavage. Peritoneal lavage was performed by infusing 20 ml Krebs-Ringer-Hepes (KRH) through a 3 cm midline incision into the peritoneal cavity and subsequent flushing of the abdomen above a 50 ml centrifuge tube. The abdominal organs were two to three times flushed with KRH.

Microcapsules were either freely floating and non-adherent, or adherent to the surface of abdominal organs. First, non-adherent microcapsules were retrieved by peritoneal lavage. Subsequently, the micro-

capsules adherent to the surface of abdominal organs, were excised and processed for histology.

NIH guidelines for the care and use of laboratory animals have been observed. All surgical procedures were performed under halothane anesthesia.

2.7. Microscopy

To assess overgrowth after implantation, samples of adherent capsules recovered by excision and non-adherent capsules were fixed in pre-cooled Bouin-solution, buffered with 0.05 M phosphate in saline (pH 7.4), and processed for paraffin embedding. Sections were prepared at 2 μ m and stained with hematoxylin and eosin (H&E) and applied for detecting capsules with and without overgrowth.

To confirm the integrity of capsules before and after implantation, samples of capsules were meticulously inspected for the presence of irregularities or broken parts in the capsule membranes by light-microscopy (Leica, Hamburg, Germany).

2.8. Diffusion studies

The release of dexamethasone from alginate-polylysine-alginate microcapsules was determined by High Performance Liquid Chromatography (HPLC) over a period of 14 days after co-encapsulation. A sample of 100 capsules were cultured in a 5 ml volume. Samples of cultured dexamethasone-capsules were frozen at -20 °C until measurement. The culture medium was replaced every second day by fresh medium.

2.9. Statistical analysis

Data obtained from the proliferation-assays are presented as mean \pm standard deviation. Statistics used are the Wilcoxon test for non-parametric data. A $p < 0.05$ was considered to be significant different.

3. Results

3.1. Release and biological activity of dexamethasone

As shown in Fig. 1, dexamethasone can readily pass the capsule membrane and diffuse into the culture medium. The capsules do release 40 μ g/ml per 100 capsules in 48 hours. This corresponds to 0.2 μ g per capsule per 24 hours. This release remained at virtually the same level in the first week and decreased sharply in the period thereafter. At two weeks after encapsulation only 50% of the initial amount of dexamethasone was released. In some experiments we also assessed the release after four weeks and found no measurable amounts of dexamethasone.

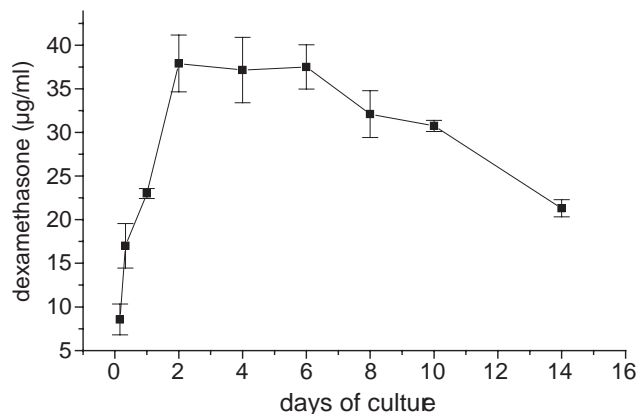


Fig. 1. Diffusion of dexamethasone from alginate-PLL-alginate microcapsules as measured by HPLC. Peak release ($36.2 \pm 5.4 \mu\text{g/ml}$) is obtained at day 2 and remained at virtually the same level in the first week, with a sharply decrease in the period thereafter.

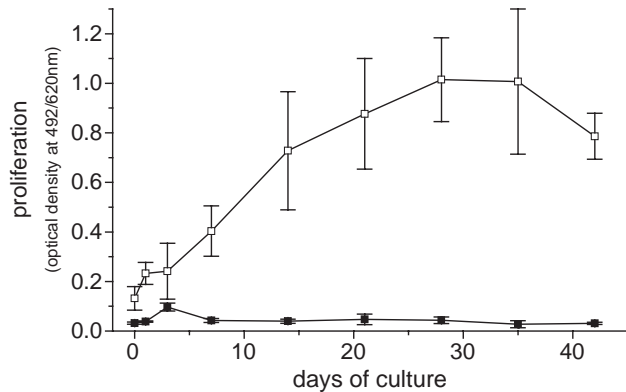


Fig. 2. Proliferation of L929-fibroblasts in conventional (open boxes) and dexamethasone (closed boxes) alginate-PLL-alginate microcapsules over a period of 6 weeks. Proliferation was measured by assessing the optical density (OD) of reduced formazan in a MTS/PMS-assay. Proliferation is strongly suppressed by co-encapsulation with dexamethasone; peak-OD (conventional capsule): 1.015 ± 0.169 at day 28; peak-OD (dexamethasone capsule): 0.097 ± 0.016 at day 3 ($p < 0.05$).

Since reportedly many agents with biological functions lose their activity after prolonged periods of culture, we also measured the biological activity of the dexamethasone. Another reason is to confirm that dexamethasone maintains its function after implantation of the dexamethasone-containing capsules.

The activity was determined by assessing the anti-proliferative effect of dexamethasone on fibroblasts. We chose fibroblasts since it has been shown that these cells show a dose dependent gradual reduction in proliferative activity after exposure to the steroid [21].

As shown in Fig. 2, L929-mouse-fibroblasts maintain their ability to proliferate in the alginate-PLL capsules as illustrated by an increase in formazan breakdown in the first 28 days after implantation. This proliferation was significantly ($p < 0.05$) inhibited in the dexametha-

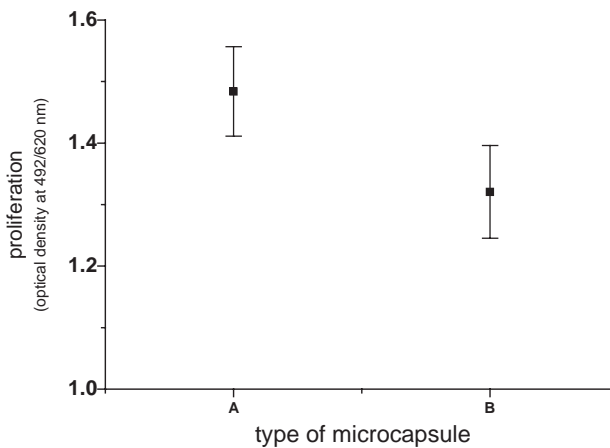


Fig. 3. Proliferation of unencapsulated L929-fibroblasts in culture with conventional (A) and dexamethasone (B) alginate-PLL-alginate microcapsules, assessed by reading optical density (OD) of reduced formazan in a MTS/PMS-assay. Proliferation is significantly inhibited by culture with dexamethasone capsules; (A) OD: 1.484 ± 0.072 ; (B) OD: 1.321 ± 0.075 ($p = 0.008$).

sone-capsules which illustrates conservation of the biological activity of dexamethasone.

To confirm that not only the dexamethasone inside the capsule but also the steroids that have diffused to the outside of the capsule have maintained their biological activity we performed a coculture of fibroblasts with alginate-PLL capsules with and without dexamethasone. As shown in Fig. 3, the fibroblasts cocultured with the dexamethasone-capsules showed a significantly ($p < 0.05$) lower proliferation rate than the fibroblasts cocultured with empty capsules.

It is mandatory to confirm that the reduction in formazan breakdown is caused by an antiproliferative effect and not caused by an undesired, toxic effect of dexamethasone. Therefore we also performed a viability assay on the fibroblasts by application of a life-dead-assay.

L929-fibroblasts showed a similar if not identical viability of nearly 100% of the cells at day 1 after encapsulation in both the conventional and dexamethasone capsules (Fig. 4). This illustrates an absence of toxic effect of the co-encapsulated dexamethasone on the encapsulated cells. After the first day, the number of fibroblasts in conventional capsules was observed to increase considerably either as single cells or as cell-clusters (Fig. 5A and B). This corroborates our findings with formazan breakdown. This increase in number was not observed in dexamethasone capsules.

3.2. *In vivo* responses against microcapsules

Prior and after implantation, we observed all batches of microcapsules to contain some imperfect capsules. The number of imperfect capsules was small and represented not more than approximately 5% in the

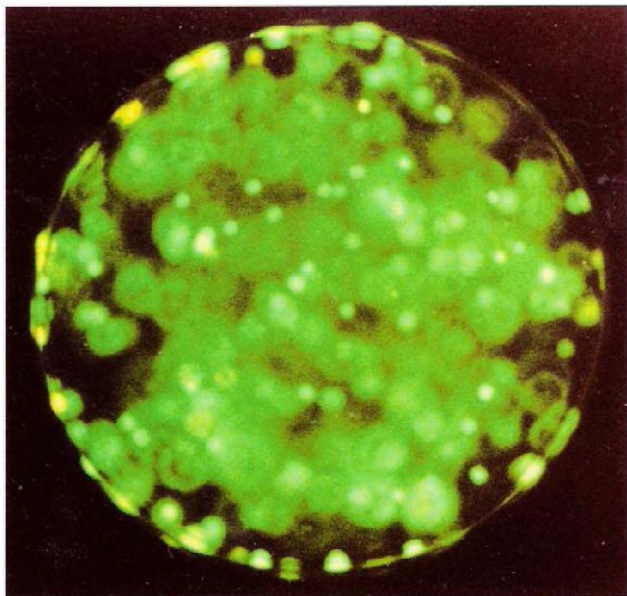


Fig. 4. Viability of fibroblasts encapsulated in dexamethasone-containing capsules. Note the absence of red coloured, dead cells (fluorescence microscopy, original magnification $100\times$).

batches of conventional capsules and dexamethasone capsules. Light microscopy showed both types of capsules to have similar degrees of integrity as illustrated by absence of strains and a surface without visible irregularities (Fig. 6A and B).

Although there was no difference in the number of imperfect capsules, the responses were not similar but always less severe against (alginate-dexamethasone)-PLL-alginate capsules than against conventional alginate-PLL-alginate capsules (Table 1). At four weeks after implantation, the majority of alginate-PLL-alginate was found to be adherent to the omentum, liver, and guts without affecting the mesothelial lining. All capsules were covered with cellular overgrowth (Fig. 7A). The overgrowth was predominantly composed of fibroblasts.

In contrast, the vast majority of (alginate-dexamethasone)-PLL-alginate capsules were not adherent to the abdominal organs but freely floating in the peritoneal cavity between the guts. The capsules could readily be flushed out of the peritoneal cavity and were virtually all free of fibrotic overgrowth (Fig. 7B).

4. Discussion

To our best knowledge, this is the first report showing a complete abrogation of the tissue response against immunisolating alginate-PLL microcapsules by co-encapsulation of immunomodulating agents. This is an essential step towards clinical application of encapsulated cells since overgrowth of the capsules is associated

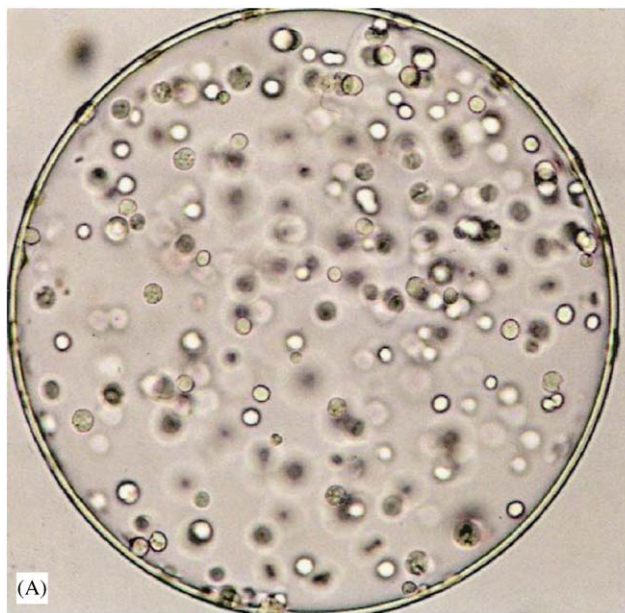


Fig. 5. Cultured encapsulated L929 fibroblasts in conventional capsules at day 1 (A) and day 14 (B) after encapsulation; the number of fibroblasts was observed to increase considerably either as single cells or as cell-clusters (light microscopy, original magnification $100\times$).

with necrosis of the cells and failure of the graft [6]. The prevention of overgrowth was accomplished by inclusion of dexamethasone in the capsules. This is a rather different and unconventional approach since most groups, including ours [5,9,11,14,22], have focussed up to now on chemical modification of the capsules to improve the biocompatibility. However, recent new insight into the tissue responses have shown that the responses against capsules are not only initiated by the capsules chemical composition but are also caused by non-specific reactions as the consequence of surgical

trauma associated with implantation of the grafts [15]. Since it will be difficult if not impossible to prevent these responses we decided to design and test approaches

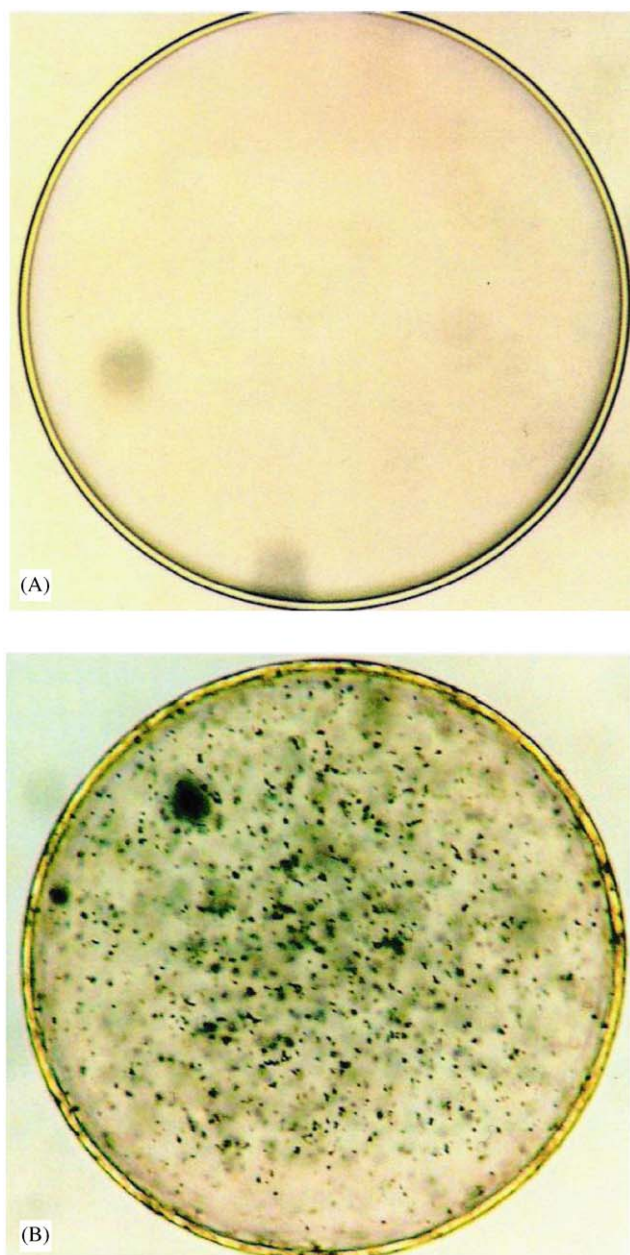


Fig. 6. Alginate-PLL microcapsules without (A) and with (B) dexamethasone before implantation. Note the absence of strains or irregularities and the small unsolved, aggregates of dexamethasone inside the capsules (light microscopy, original magnification 100 \times).

Table 1

Biocompatibility of conventional alginate-PLL-alginate capsules and dexamethasone- alginate-PLL-alginate capsules at four weeks after implantation in the peritoneal cavity of rats

Capsule type	<i>n</i>	Location intraperitoneally	Type of cells in overgrowth
Alginate-PLL-alginate	5	Adherent	Fibroblasts
(Dexamethasone-alginate)-PLL-alginate	5	Free floating	No overgrowth

which suppress the responses rather than to avoid them by changing the composition of the capsules.

We choose to apply dexamethasone to suppress the tissue responses since dexamethasone is a glucocorticosteroid with a broad suppressing effect on inflammatory agents such as IL-1-Beta, TNF-alpha, and prostaglandins [23–26]. One may argue, that dexamethasone is not applicable for all encapsulated cell approaches since dexamethasone has a reported effect on insulin resistance [27–29] and can consequently therefore not be applied in transplantation of encapsulated pancreatic islets. However, the diabetogenic effect of dexamethasone is dose dependent [30] and usually reaches an effect

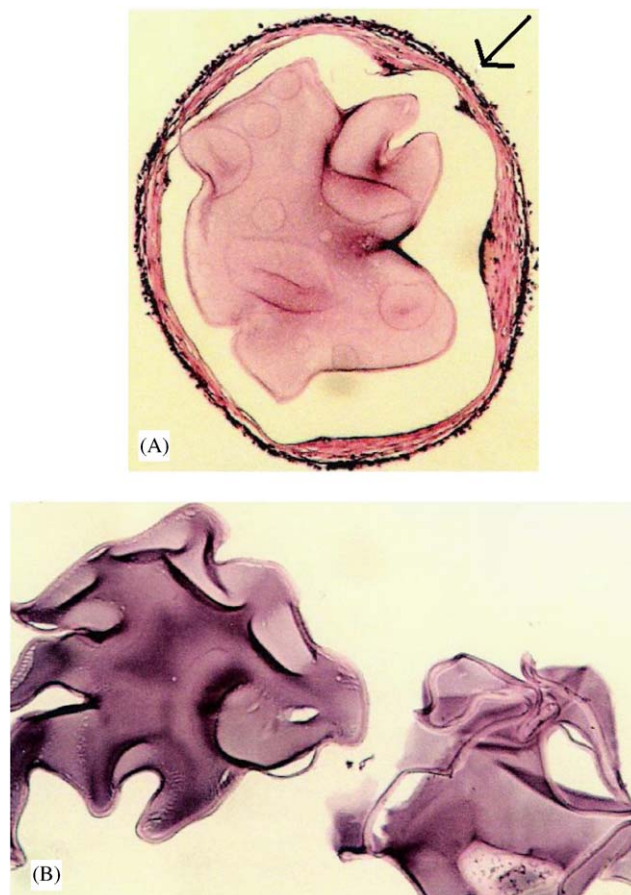


Fig. 7. Alginate-PLL microcapsules without (A) and with (B) dexamethasone after implantation. The microcapsules without dexamethasone are covered by several layers of fibroblasts (see arrow) while the dexamethasone capsules are free of overgrowth (H&E staining, LM, original magnification 100 \times).

only at far higher concentrations than applied in the present study. Also, it has to be applied chronically to cause diabetogenic effect. It is unlikely that the short-term release in the concentrations applied in the present approach will have any effect in the systemic circulation or in the peripheral tissues of the recipients. This is corroborated by the findings of Wijsman who found prolonged survival of allogenic islets in dexamethasone treated BB-rats [31].

The tissue response against capsules in the first week is composed of monocytes/macrophages, granulocytes, fibroblasts, erythrocytes, multinucleated giant cells, and basophiles [15]. These cells are in a time dependent fashion involved in the reaction against capsules and are probably regulated by cytokines secreted by macrophages which can be found in the vicinity of the grafts as soon as a few hours after implantation [15]. The response is associated with the release of bioactive proteins such as trombin and fibronectin [15] which adhere to the capsules surface and provide binding sites for the cells to overgrow the capsules. It is essential to apply immunomodulating agents which act on macrophages [23–25] since this may block virtually the whole inflammatory response which finally results in release of bioactive proteins and adherence of cells. It is conceivable that dexamethasone functions via this route. This is corroborated by the findings of Hsu et al who found a prolonged survival of encapsulated islet grafts after injection of 15-deoxy-spergualine which selectively suppress macrophage function [32].

The suppressive effect of immunomodulating agents in application of encapsulated cells may be temporary rather than permanent. It is, however, difficult to predict how long the agents have to be released. According to several groups the tissue response is complete within the first week [15,33]. However, complete engraftment of the tissue and, therefore, homeostasis in the transplantation site can take a longer period of time (take e.g. [6]). In the present study we show that a stable release of 0,2 µg dexamethasone per capsule per day during the first week and a gradual decrease of the release thereafter is sufficient to completely avoid the tissue responses against and overgrowth of the capsules until 28 days after transplantation.

In the present study, we found a more severe tissue response against conventional alginate-PLL-alginate capsules than reported by other groups [5,34]. Plausibly, this is caused by technical variations in the different studies since producing biocompatible alginate-PLL-alginate capsules is far from simple [35]. The chemical structure of alginate-PLL-alginate capsules, and thus its biocompatibility, is determined by many factors such as temperature, type and concentration of alginate (i.e. the guluronic acid content) [11], and the type of polylysine [13]. Small differences in these factors induce variations in the capsules chemical and mechanical properties

[12,13,36] and biocompatibility [11,12]. This has been the main rationale to seek for a more practical encapsulation-technologies and to design means to block the responses. The present study illustrates that the latter approach can be successful and that temporary release of immunomodulating agents can circumvent the harmful tissue response without complicated technical modification of the capsules.

5. Conclusions

In conclusion, dexamethasone in alginate-PLL capsules showed an antiproliferative effect on fibroblasts *in vitro* and provided a local anti-fibrotic effect around capsules *in vivo*. In present research efforts we will focus on the functional survival of cells enveloped in capsules containing dexamethasone. These strategies will serve as pertinent basis for future clinical application of immunoisolated cells.

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