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Production of purified alginates suitable for use in immunoisolated transplantation

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Abstract. Alginate is used as a matrix for immunoisolation of cells and tissues in vivo. We have demonstrated previously that commercial alginates contain various fractions of mitogenic impurities and that they can be removed by free flow electrophoresis. The use of purified material is a necessity in order to reveal the parameters that control biocompatibility of the implanted material (such as stability, size, surface charge and curvature, etc.). In this study, we present a protocol for the chemical purification of alginates on a large-scale. Beads made from alginates purified by this multi-step chemical extraction procedure did not induce a significant foreign body reaction when implanted for 3 weeks either intraperitoneally or beneath the kidney capsule of Lewis or non-diabetic BB/Gi rats.

Introduction

Immunoisolation by means of capsules based on alginate as a matrix offers a very promising strategy to overcome the host immune response that is observed when transplanting xenografts (Lim and Sun 1980). Microencapsulation of hormone-producing cells (and tissues) in Ca^{2+}- and Ba^{2+}-cross-linked alginates has been used for the treatment of diabetis mellitus (Geisen et al. 1990; Calafiore 1992; Zekorn et al. 1992b), liver and parathyroid diseases (Darqui and Sun 1987; Trompkins et al. 1988; Cai 1989). However, experiments in animal models have shown (Cole et al. 1989; Mazaheri et al. 1991) that foreign-body reactions occur after implantation of alginate-based capsules. These may result in failure of the cellular functions of the transplants some time after in-vivo application.

Otterlei et al. (1991) have concluded from their results that the adverse processes are associated with mitogenic properties of the mannuronic acid component of the alginates. These authors recommended the use of alginates containing a high proportion of guluronic acid for implantation studies, despite their poor permeability (Klein et al. 1983). Recently, we have demonstrated (Zimmermann et al. 1992) that commercial alginates contain various fractions of impurities that exhibit mitogenic activity in an in-vitro test. Removal of these contaminants by free-flow electrophoresis (FFE) resulted in alginate preparations that did not provoke foreign-body reactions after cross-linkage with Ba^{2+} ions, at least 3 weeks after implantation into the peritoneal cavity of rodents (Zimmermann et al. 1992).

Purification of commercial alginates by FFE has the disadvantage that highly sophisticated, expensive equipment is needed, and that the procedure is very time-consuming, in particular when alginate must be purified on a large scale. In this communication we report on a multi-step, chemical extraction procedure that allows the large-scale production of purified alginates. In in-vivo experiments the products exhibited similar properties to those of the FFE-purified alginates.

Materials and methods

Alginates. The following commercial alginates were used: Manugel GHB (Lot 548643, 562511 and 529061), Manucol DH, Manucol LB, Manugel DMB, Kelcogel LV, Kelgin LV (all from Kelco, London, UK), alginic acid (Sigma, Taufkirchen, Germany) and alginic acid (Roth, Karlsruhe, Germany).

The alginates were usually dissolved in distilled water (1% w/v) and subsequently filtered through a 0.2-μm pore size filter.

Assays for mitogenic activity. To enable rapid screening of the mitogenic activity of the various alginate fractions obtained during and after the purification procedure, in-vitro assays based on murine splenocytes were used. To this end, 10 μl of the filtered alginates were filled into the wells of a 96-well plate (Greiner, Nürtingen, Germany) under sterile conditions.

Splenocytes were prepared from female Balb-c mice (8–10 weeks old) as described elsewhere (Zimmermann et al. 1992). The cells were suspended at a suspension density of $1 \cdot 10^6$ ml^{-1} in complete growth medium (CGM) consisting of RPMI 1640 medium supplemented with 10% foetal calf serum (Boehringer, Mannheim, Germany), 2 mM L-glutamine, 2 mM sodium pyruvate, 1× non-essential amino acids (Boehringer, Mannheim,

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Germany), 50 μM 2-mercaptoethanol, 100 units penicillin ml^{-1} and 100 μg streptomycin ml^{-1} (Biochrom, Berlin, Germany). Then, 100 μl of this suspension was added to the alginate samples. In all wells the cells were grown for 3–5 days at 37° C in a 5% CO_2-supplemented atmosphere. Five microlitres of a 0.5% Trypan blue solution was added to 45 μl cell suspension and the intact blasts (i.e. those large-sized splenocytes that excluded the dye) per well were counted by using a Neubauer chamber.

Counting the stimulated lymphocytes with a haemocytometer has significant advantages over other assays for cell proliferation, for example, Coulter counter, ^{3}H-thymidine incorporation or the 3,(4,5-dimethyl-thiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, because it simultaneously provides both qualitative and quantitative data on culture morphology and cell growth response (Turner et al. 1989). However, this method is very time-consuming and the number of samples that can be processed is rather limited. Therefore, in a parallel set of experiments the activated lymphocytes were determined by the MTT assay. This rapid and precise colorimetric assay is based on the observation that cells with intact mitochondrial respiration can convert the membrane-permeable yellow tetrazolium salt MTT into a water-insoluble blue product (Mosmann 1983; Hansen et al. 1988).

To 100 μl of lymphocytes, seeded into the wells of 96-well plates (Greiner), 20 μl of an MTT solution (5 mg ml^{-1} dissolved in phosphate buffered saline, Sigma) was added. After 3 h at 37° C, 100 μl isopropanol containing 0.04 M HCl was added. The cells were mixed rigorously for about 10 min and the absorption of each sample was measured at 570 nm and 650 nm (Thermomax microplate reader, Molecular Devices, Menlo Park, Calif., USA). Lymphocyte proliferation was determined after calibration of the absorption difference ($E_{570\,nm}$-$E_{650\,nm}$) versus the number of lipopolysaccharide (LPS)-activated lymphocytes determined by means of the haemocytometer.

The sensitivity and accuracy of both assays were tested by the addition of LPS to the lymphocytes. As shown in Fig. 1, both tests gave comparable results in the concentration range 0.1–5 μg LPS ml^{-1}.

Endotoxin assay. Endotoxin was determined by a commercial *Limulus*-lysate assay (Scheer 1988) following the protocol of the E-Toxate kit recommended by Sigma. Alginate samples (1% w/v) were diluted with pyrogen-free water (ratio 1:100). The wells of a 96-well plate were filled with 40 μl alginate solution, 40 μl *Limulus*-lysate reagent were added and the plates were incubated at 37° C in an temperature-controlled microplate reader (Thermomax, Molecular Devices). The clotting reaction was followed by measurement of the absorption at 340 nm for 1 h.

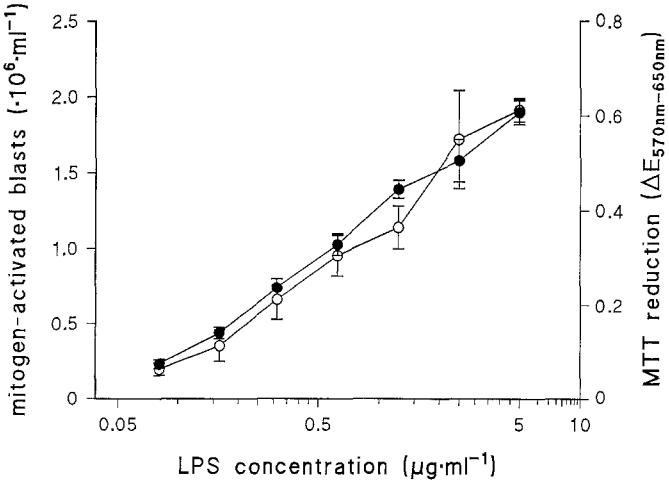


Fig. 1. Detection of mitogenic activation of murine splenocytes in vitro. Murine splenocytes (prepared from 8-week-old female Balb c mice) were cultured for 3 days in the presence of different concentrations of bacterial lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B12). After 3 days, activation was measured either by counting of blasts in a haemocytometer (○) or by 3,(4,5-dimethyl-thiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction (●)

Fluorescence spectroscopic analysis of alginates. Fluorescence measurements were performed with an LS50 luminiscence spectrometer (Perkin Elmer, Beaconsfield, UK) following the protocol of Skjaek-Braek et al. (1989). The alginate samples were dissolved in distilled water (1% w/v) and filtered through a 0.2-μm pore size membrane filter (Schleicher & Schuell, Dassel, Germany). Using 366 nm fluorescence excitation, emission spectra were recorded between 380 nm and 600 nm. The spectra were analysed using the fluorescence data manager software supplied by Perkin Elmer.

Implantation of Ba^{2+}-cross-linked alginate beads. The preparation of Ba^{2+}-cross-linked alginate beads has been described in detail elsewhere (Zekorn et al. 1992a). Briefly, alginate purified from Manugel GHB was dissolved in a 0.9% NaCl solution (2% w/v), injected through the central channel (outer diameter 0.5 mm) of a home-made nozzle and allowed to fall into a 20 mM $BaCl_2$ solution. This resulted in the formation of beads with a mean diameter of approximately 600 μm. These large-sized beads were used for intraperitoneal (i.p.) implantation. Upon application of a higher coaxial air flow, beads with a mean diameter of 50–200 μm were obtained that were suitable for implantation beneath the kidney capsule. After several washings in 0.9% NaCl solution, the beads were incubated for 1 day in RPMI 1640 medium, supplemented with 10% foetal calf serum (Gibco Europe, Germany) to imitate the conditions of the transplantation procedure of encapsulated islets of Langerhans in diabetic rats (Zekorn et al. 1992a).

The beads were implanted by lateral laparatomy under ether anaesthesia into the peritoneal cavity or beneath the kidney capsule of non-diabetic BB/Gi female rats ($n=5$, University of Giessen) and Lewis rats ($n=5$, body weight 250–270 g, Charles River Wiga, Sulzfeld, Germany). After 3 weeks, the animals were sacrificed. Nephrectomy was performed and the i.p. implanted beads were removed from the peritoneal cavity by lavage and peritoneal biopsy.

The beads from the peritoneum were immobilized in a fibrin clot. Both the fibrin-embedded beads, as well as the explanted kidneys were Bouin-fixed, and paraffin-embedded. Serial sections were stained with hematoxilin-eosin and Masson-Goldner and examined under the microscope.

Results

Procedure for alginate purification

The affinity of Ba^{2+} ions for alginate is much higher than that of Ca^{2+} ions (Dainty et al. 1986; Tanaka and Irie 1988; Schnabl and Zimmermann 1989), which are usually used for the cross-linkage of alginate in immobilisations studies (Klein et al. 1983; Rehm and Reed 1987). Therefore, Ba^{2+}-alginate gels (in contrast to Ca^{2+}-alginate gels) are very stable in acid and neutral solutions containing chelating agents such as citrate, phosphate or ethylenediaminetetraacetic acid (EDTA). However, we found that chelating agents can dissolve the Ba^{2+}-cross-linked alginates very easily and gently in strongly alkaline solutions. These properties of Ba^{2+}-alginates were used for removal of mitogenic contamination from the raw material.

The contaminants were eluted by treatment of Ba^{2+}-alginate beads with solutions using different

agents followed by ethanol extraction. Next, the beads were dissolved in strongly alkaline, EDTA-containing solutions. The viscous alginate solution was then subjected to dialysis (in order to remove the Ba^{2+} ions and the reagents) and finally the Na^+-alginate precipitated by the addition of ethanol. Purified alginates of high and reproducible quality were obtained when the commercial alginates were subjected to filtration and treatment with charcoal before the chemical purification procedure.

Screening experiments showed that the experimental conditions should be closely adjusted to the following protocol in order to obtain 1–2 g of purified alginate independent of the ratio of mannuronic to guluronic acid of the original material.

Commercial alginate (18 g) was dissolved in 1.2 l of distilled water and 18 g charcoal SP1 (Serva no. 11416, Heidelberg, Germany) was added. The mixture was stirred for 3–4 h with a magnetic stirrer and subsequently filtered through a cellulose nitrate membrane filter (Sartorius, Göttingen, Germany) of decreasing pore sizes (0.8 μm, 0.45 μm and 0.2 μm, respectively). The filtered alginate solution was forced through a jet head into 4.5 l of a 50 mM $BaCl_2$ solution to produce beads of about 1.5 mm diameter.

The polymerization process was completed after about 20 min. The supernatant was then removed using a metal sieve (1-mm mesh) and the beads were washed extensively with distilled water. Afterwards the beads were transferred to 4.5 l of 1 M acetic acid, pH 2.3. After incubation for 14 h the solution was removed and the beads were washed again with distilled water. This acid extraction step was repeated twice.

The beads were resuspended in 4.5 l of a 500 mM sodium citrate solution, pH 8.0. The citrate solution was changed twice every 7–8 h. After this extraction step the beads were once again washed carefully with distilled water.

Next, the beads were extracted twice for 16 h each time with 5 l of 50 and 70% ethanol (containing 5% acetone), respectively. Then the beads were subsequently washed with distilled water, with a 20 mM $BaCl_2$ solution and again extensively with distilled water.

For recovery of the Na^+-alginate the beads were dissolved in 1 l of a 250 mM alkaline EDTA solution, pH 10.0 and kept overnight. The viscous solution was filtered through a 0.2-μm cellulose nitrate membrane filter and was dialysed (Medicell dialysis tubing, MWCO 12–14 kDa, Roth, Karlsruhe, Germany) for up to 20 h against demineralized water. After addition of 10 mM NaCl, the purified alginate was precipitated by ethanol and dried under sterile conditions.

Fluorescence spectroscopic analysis

Figure 2 shows typical fluorescence spectra recorded for 1% (w/v) solutions of Manugel GHB in various stages of the purification process. The raw material shows high intensity fluorescence between 400 and 600 nm with a maximum at 420 nm and a broad peak at 445 nm. According to Skjaek-Braek et al. (1989) the peak at 420 nm corresponds to the Raman band of water. Charcoal treatment of raw alginate dramatically reduced the fluorescence maximum at 445 nm from 76 arbitrary fluorescence units (raw alginate) to 18 arbitrary fluorescence units. Further reduction of the fluorescence maximum at 445 nm to 9 arbitrary fluorescence units was observed in the purified alginate after the extraction procedure. However, the fluorescence spectrum of the purified product still differs substantially from those of the solvent.

In-vitro and in-vivo mitogenic activity of alginates purified chemically

Figure 3 shows the mitogenic activity before chemical purification of samples from some commercial alginates (1 mg ml^{-1}) with various mannuronic/guluronic acid ratios. The highest mitogenic activity was found in the alginates Manucol DH, Manucol LB, Manugel GHB and Kelgin LV. Medium mitogenic activity was found in samples from Kelcogel LV, Manugel DMB and the alginic acids (Roth and Sigma). It should be noted that the mitogenic activity of the raw alginates apparently is not correlated to the ratio of mannuronic to guluronic acids.

There are good reasons (Zimmermann et al. 1992) to assume that (at least part of) the mitogenic activity of raw alginates may be caused by endotoxins (e.g. LPS). However, the endotoxin content of raw Manugel GHB as measured by the *Limulus*-lysate assay (Fig. 4) was only 30 ng LPS mg^{-1} alginate (corresponding to 140 endotoxin units mg^{-1}). The data shown in Figs. 1 and 3 suggest that the mitogenic activity of 1 mg crude

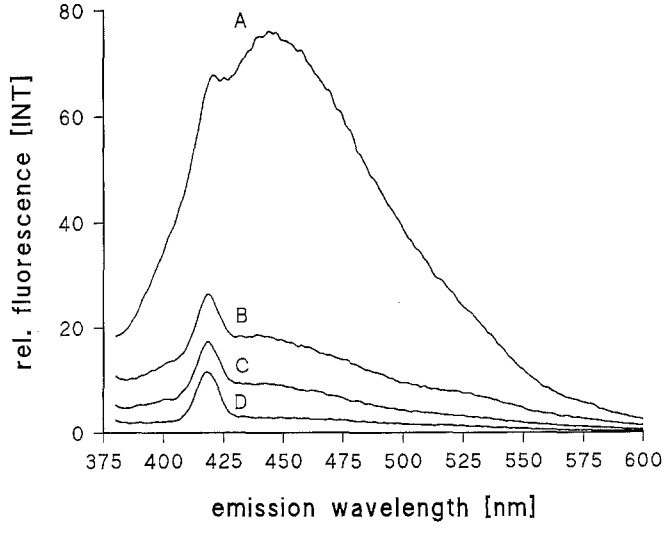


Fig. 2. Fluorescence spectroscopy of alginates. Samples of Manugel GHB in various stages of the purification process were dissolved in distilled water (1%, w/v) and filtered through 0.2-μm membrane-filters. Fluorescence was excited at 366 nm and emission spectra were recorded between 380 and 600 nm: *A*, raw Manugel GHB; *B*, Manugel GHB after charcoal treatment; *C*, Manugel GHB after the chemical purification procedure (charcoal treatment omitted); *D*, solvent (water): INT, arbitrary fluorescence units

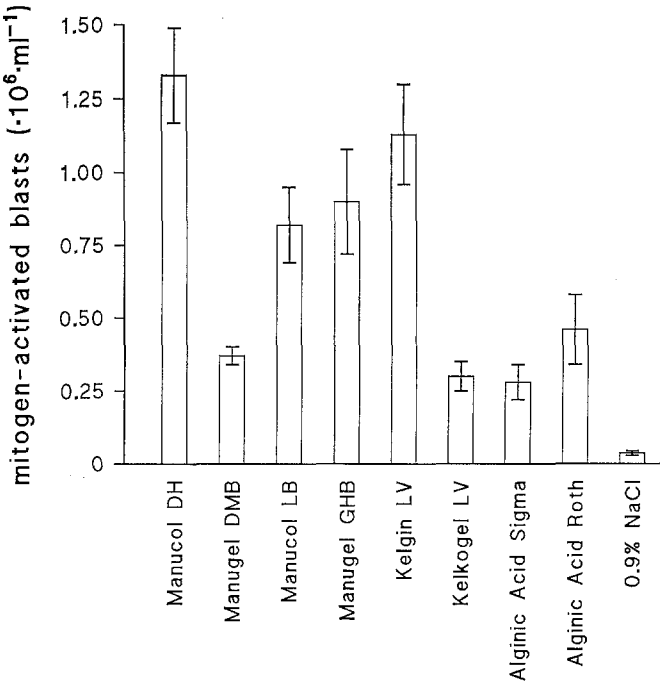


Fig. 3. Mitogenic activity of different commercial alginates. Murine splenocytes (prepared from 8-week-old female Balb c mice) were cultured for 3 days in the presence of 1 mg·ml^{-1} of different alginates. *Histograms* represent the number of mitogen-activated blasts as counted in a haemocytometer (±SD, *bars*)

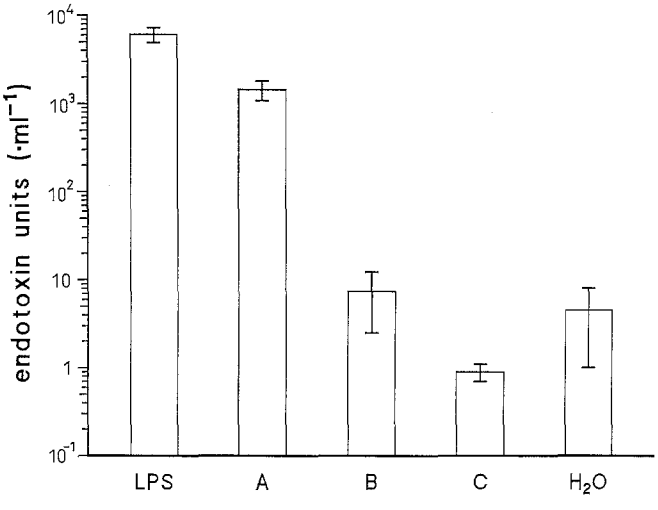


Fig. 4. Endotoxin content of crude and purified Manugel GHB. Manugel GHB was purified by the extraction method. The endotoxin content of 1% (w/v) solutions of the alginate was measured in 1/100 dilution by a commercial *Limulus*-lysate test kit (E-Toxate assay, Sigma, Taufkirchen, Germany). The LPS (1.6 μg·ml^{-1}) was from *E. coli* serotype 055:B12. *Histograms* represent the endotoxin content of different alginate samples (±SD, *bars*): *A*, raw Manugel GHB; *B*, Manugel GHB after charcoal treatment; *C*, Manugel GHB after the chemical purification procedure (charcoal treatment omitted)

Manugel GHB is about equal to that of 1 μg LPS. It can be concluded that LPS is responsible for only about 3% of the total mitogenic activity of raw alginates (Manugel GHB).

After chemical purification, the endotoxin content of the alginates was nearly zero (Fig. 4). The mitogenic activity of all commercial alginates was greatly diminished, but still higher than the control performed in electrolyte solution when liquid alginate solutions were used (Fig. 5a). In contrast to cross-linking with Ca^{2+} ions (data not shown), cross-linking of the alginate with Ba^{2+} ions resulted in a further reduction of mitogenic activity nearly to the control level (Fig. 5b).

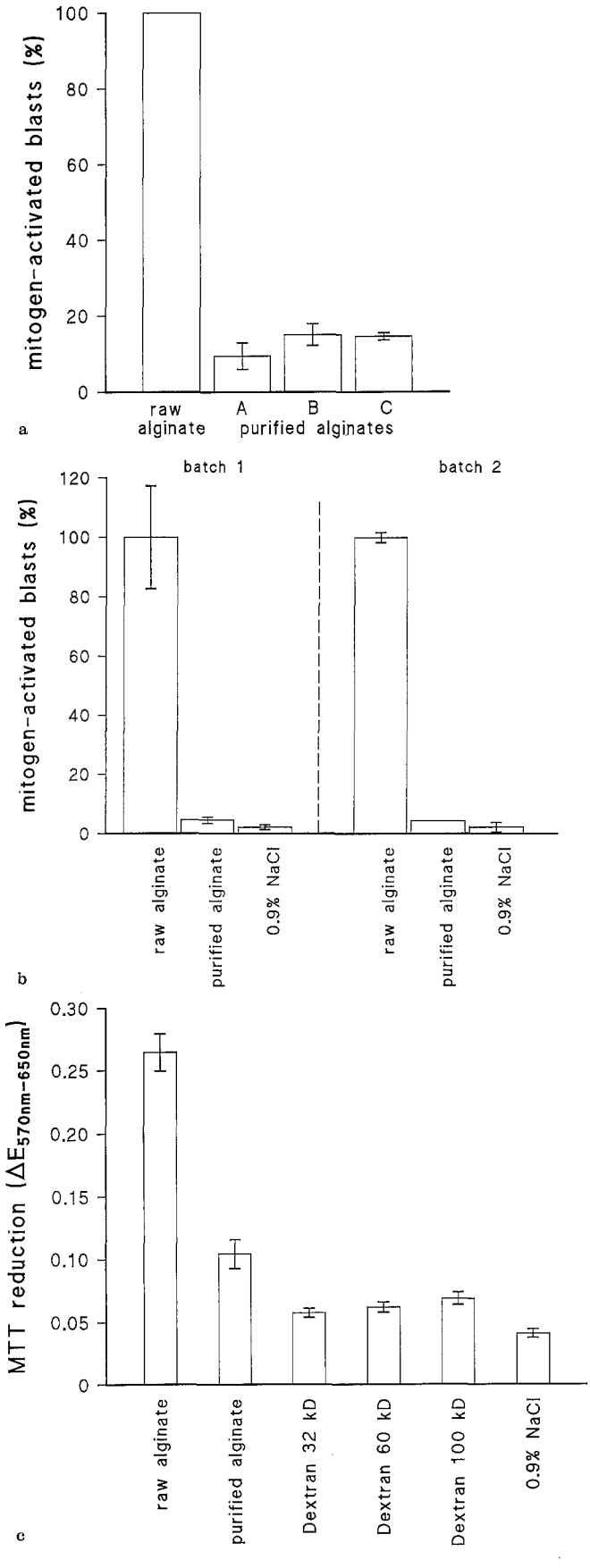
The MTT assay always gave somewhat higher values of mitogenic activity than the haemocytometer assay. Since stimulation of the lymphocytes with LPS led to comparable results (Fig. 1) it cannot be excluded that the viscosity (and the high charge) of the alginate solutions interfered with the assays (see also Simpson et al. 1988). To test this, 1 mg dextrans ml^{-1} of various molecular mass were added to the control solution. The viscosities of these solutions (1.43–1.44 cP) were less than that of alginate solutions (Manugel GHB 1% in culture medium 2.51–2.76 cP). Despite the fact that dextrans show only a very low mitogenic activity (if any), it is evident from Fig. 5c that dextrans with a molecular mass of about 100 kDa also led to positive mitogenic results in both assays. This shows that the information about mitogenic activity that can be derived from such in-vitro assays is limited. Such tests are apparently useful for rapid screening of the efficiency of the purfication process, but cannot completely replace the crucial animal experiment.

Indeed, when Ba^{2+}-cross-linked, empty (purified) alginate beads were implanted for 3 weeks into non-diabetic BB/GI- or Lewis-rats (Fig. 6a, b) fibrotic or inflammatory reactions were observed only occasionally. On average, 1–5% of the beads made from purified alginate showed a foreign body response when implanted intraperitoneally. The rate was even less when the beads were located beneath the kidney capsules.

Discussion

The results reported here demonstrate that mitogenic contaminants and endotoxins can apparently be removed from raw alginates by chemical extraction. The biocompatibility of the purified alginates was apparently independent of the transplantation site (kidney capsule or peritoneal cavity). For therapy, however, transplantation of microencapsulated islets beneath the kidney capsule is to be preferred to peritoneal transplantation. In contrast to peritoneal implants, the microencapsulated islets implanted beneath the kidney capsule remain localized and may be resected in case of complications (Zekorn et al. 1992b). The peritoneal cavity also has the disadvantages of low pO_2 and active macrophages (Zekorn et al. 1992b).

The possible occurrence of fibrotic overgrowth observed around some beads remains to be studied. However, it must be noted that any (long-term) instability or changes in the size of the beads, as well as any imperfection in the process of bead production affecting peripheral surface smoothness can influence the



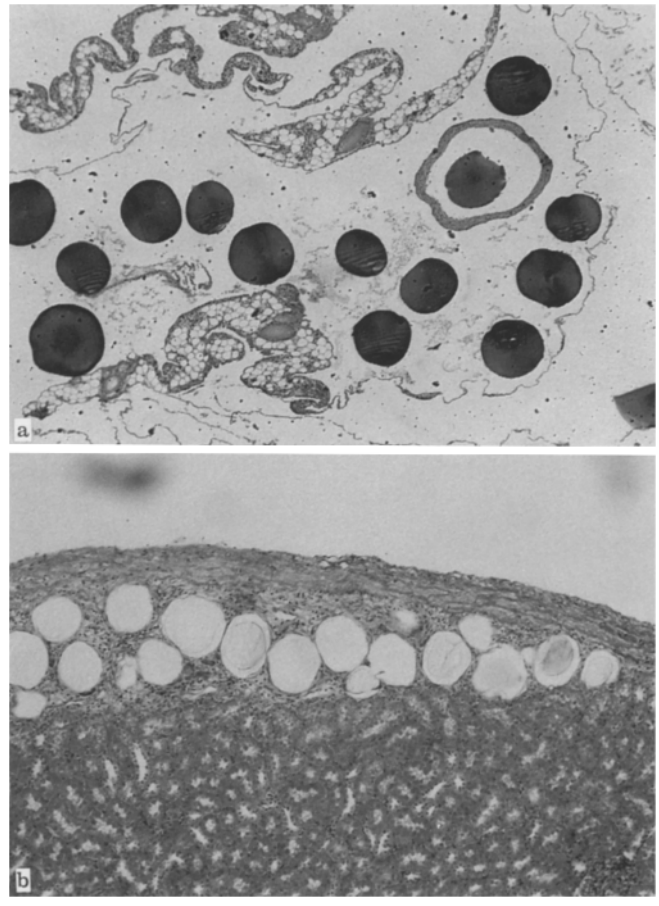


Fig. 6a, b. Barium-cross-linked alginates after 3 weeks implantation in non-diabetic BB/Gi rats. Alginate beads made up from purified Manugel GHB: **a** peritoneum (Haematoxilin-Eoxin-staining, ×100); **b** beneath the kidney capsule (Masson-Goldner-staining, ×100). Similar results were obtained after implantation in Lewis rats

Fig. 5. a Effect of the purification procedure on the mitogenic activity of *liquid samples* of Manugel GHB. Murine splenocytes were cultured for 3 days in the presence of 1 $mg \cdot ml^{-1}$ of a liquid sample of the filtered alginates. *Histograms* represent the percentage number of mitogen-activated blasts as counted in a haemocytometer (±SD, *bars*). The three purified alginate samples represent different batches of the extraction. Each batch was measured at least twice. **b** Effect of the extraction procedure on the mitogenic activity of two different batches of Ba^{2+}-*cross-linked samples* of Manugel GHB. 50 µl of the alginate solutions (1%, w/v) were polymerized in the wells of a 96-well plate by 20 mM solution of $BaCl_2$, respectively, and subsequently were washed extensively with saline and with culture medium. Murine splenocytes (100 µl) were cultured for 3 days in the presence of the polymerized alginate samples. *Histograms* represent the percentage of mitogen-activated blasts as counted in a haemocytometer (±SD, *bars*). **c** Apparent mitogenic activity of raw and extracted Manugel GHB compared to pyrogen-free dextrans. Murine splenocytes were cultured for 3 days in the presence of 1 $mg \cdot ml^{-1}$ of a liquid sample of the filtered alginates and dextrans (Serva, Heidelberg, Germany). *Histograms* represent the formation of the blue formazan dye induced by viable splenocytes in the MTT assay (±SD, *bars*)

properties of the material under in-vivo conditions (Lum et al. 1992).

The analysis of the purified alginates by means of fluorescence spectroscopy showed the continued presence of residues that exhibited emission at 445 nm. These contaminants could not be identified. However, the animal studies showed that these impurities did not initiate a foreign body reaction – at least, when the alginate was cross-linked with Ba^{2+} ions.

In contrast to FFE, the chemical procedure for purification of alginates can easily be scaled up in order to meet the demands of materials for transplantation studies in animals and for possible therapy. Time and costs of the chemical purification process would be reduced if it were possible to combine the procedure presented here with the industrial process for the extraction of alginates from brown seaweed.

A very important advantage of the chemical purification procedure is that it can be applied (with only minor adjustments of incubation times in the different extraction media) to alginates with various ratios of mannuronic acid to guluronic acid. Thus, alginate capsules with either high mannuronic acid or high guluronic acid contents can be used for applications such as transplantation (Clayton et al. 1991).

Purification of alginates is the first important step in the development of microcapsules for transplantation of hormone-secreting cells and tissues. Up to the present, progress in the field was hampered by (often varying and irreproducible) inflammatory and foreign-body reaction against the implant (Mazaheri et al. 1991; Cole et al. 1989) making it impossible to develop standard protocols for implantation. Further studies must be directed to the development of alginate capsules with optimized diffusional properties, size, surface curvature and stability. The aim must be capsules that provide immunoisolation of the implanted cells and allow sufficient oxygen and nutrient supply as well as efficient excretion of toxic intracellular compounds into the surroundings.

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