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The role of pathogen-associated molecular patterns in inflammatory responses against alginate based microcapsules

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Alginate-based microcapsules are used for immunoisolation of cells to release therapeutics on a minute-to-minute basis. Unfortunately, alginate-based microcapsules are suffering from varying degrees of success, which is usually attributed to differences in tissue responses. This results in failure of the therapeutic cells. In the present study we show that commercial, crude alginates may contain pathogen-associated molecular patterns (PAMPs), which are recognized by the sensors of the innate immune system. Known sensors are Toll-like receptors (TLRs), NOD receptors, and C-type lectins. By using cell-lines with a non-functional adaptor molecule essential in Toll-like receptor signaling, *i.e.* MyD88, we were able to show that alginates signal mainly *via* MyD88. This was found for low-G, intermediate-G, and high-G alginates applied in calcium-beads, barium-beads as well as in alginate–PLL–alginate capsules. These alginates did stimulate TLRs 2, 5, 8, and 9 but not TLR4 (LPS receptor). Upon implantation in rats these alginates provoked a strong inflammatory response resulting in fibrosis of the capsules. Analysis demonstrated that commercial alginates contain the PAMPs peptidoglycan, lipoteichoic acid, and flagellin. By applying purification procedures, these PAMPs were largely removed. This was associated with deletion of the inflammatory tissue responses as confirmed by an implantation experiment in rats. Our data also show that alginate itself does not provoke TLR mediated responses. We were able to unravel the sensor mechanism by which contaminants in alginates may provoke inflammatory responses.

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1. Introduction

Immunoisolation by encapsulation of therapeutic cells has been proposed as a potential therapy for a variety of diseases [1–4] in which regulation of metabolites on a minute-to-minute basis is required. The most commonly applied procedure for immunoisolation is encapsulation of tissues in alginate-based capsules as originally described by Lim and Sun [5]. Alginate, the main component of the capsule, is a polysaccharide composed of different amounts of mannuronic acid (M-chains) and guluronic acid (G-chains) linked in blocks (MM-blocks, GG-blocks and MG-blocks). Binding the molecules with multi-valent cations, such as Ca^{2+} or Ba^{2+}, forms alginate beads [6]. These beads can subsequently be coated with polyaminoacids such as poly-L-lysine to form capsules [7]. Capsules with specific physical and chemical properties [1,8] can be obtained by varying the type and concentration of the cations and the polyaminoacids. During recent years, important advances have been made with this technology. New capsule types have been developed and tested [9,10]. Human trials have been started which have shown the principle applicability of microencapsulation for the treatment of thyroid disorders [11] and diabetes type 1 [12,13]. Although successful, these studies have also shown a major hurdle that has to be overcome. Graft survival was never permanent and varied considerably from several days to months [14]. This variation in survival rate is considered to be the consequence of differences in the tissue responses (*i.e.* biocompatibility) against the applied capsules.

Many have pointed towards impurities in alginate as the major cause of the variations in success of the capsules [15–17]. Purification of alginate has been reported to reduce or delete the responses but many groups have difficulties in reproducibly producing ultrapure alginates [6,18,19]. Surprisingly, in spite of a decade of intensive research, not many have reported on the molecules that have to be removed from the alginate. Also up to now, there are to the best of our knowledge no reports on the mechanisms by which impurities contribute to inflammatory responses. We hypothesize that pathogen-associated molecular patterns (PAMPs) might be impurities present in the alginates and a dominant factor in inducing inflammatory responses following implantation of encapsulated tissues or cells. PAMPs are small molecular motifs found on groups of pathogens. PAMPs are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) on cells of the innate immune system [20]. PAMPs activate the innate immune response with the aim to protect the host from infections by deletion of pathogenic bacteria. Lipopolysaccharide (LPS), an endotoxin found on the bacterial cell membrane, is a classical example of a PAMP. LPS binds to TLR4 and subsequently activates the immune system [21]. There are many more examples of PAMPs that bind to

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different types of PRRs that might be involved in responses against alginate-based capsules.

To test the hypothesis of the involvement of PAMPs in responses against alginate based capsules a number of specific experiments were performed. First we implanted capsules composed of three different types of alginates in rats to study the tissue response against the capsules. Next we studied whether these different types of alginates were recognized by PRRs and whether these PRRs were TLRs followed by the identification of which TLRs are involved. In these experiments, next to the alginates in solution, alginates were also tested in three commonly applied encapsulation systems *i.e.* calcium-beads, barium-beads, and alginate–poly-L-lysine–alginate (APA) capsules. Finally we determined which PAMPs could be found in alginates and have to be held responsible for the responses against alginates. This was done *in vitro* and *in vivo*. Our results provide new insight in the mechanisms of responses against alginate based capsules and contribute to a better understanding of which components should be removed from alginate to reproducibly produce ultra-pure alginates and prevent inflammatory responses against alginate-based capsules.

2. Materials and methods

2.1. Graft recipients

Male inbred Albino Oxford (AO/G) rats served as recipients of capsules and were obtained from the Central Animal Laboratory of Groningen. Their body weights ranged from 300 to 350 g. NIH guidelines for the care and use of laboratory animals have been observed. All experiments were approved by the Groningen ethical commission.

2.2. Chemicals

Alginates of different compositions were obtained from ISP Alginates Ltd UK. Three types of alginates have been applied: low-G (21% G-chains, 79% M-chains, 15% GG-chains, 14% GM-chains, 57% MM-chains) [22], 2) intermediate-G (44% G-chains, 56% M-chains, 23% GG-chains, 21% GM-chains, 37% MM-chains) and 3) high-G (67% G-chains, 33% M-chains, 54% GG-chains, 13% GM-chains, 21% MM-chains). Alginates were being applied in its crude form and purified form. The composition of alginate was studied by nuclear magnetic resonance (NMR).

A 0.1% solution of poly-L-lysine (PLL) (poly-L-lysine-HCl, M_w 22 kDa, Sigma-Aldrich, The Netherlands) was sterilized by 0.2 μm filtration (Corning®, NY, USA).

QUANTI-Blue™ (InvivoGen, Toulouse, France) is a medium with a colorimetric enzyme used to detect activity of any alkaline phosphatase. QUANTI-Blue™ medium turns purple-blue color in the presence of alkaline phosphatase (SEAP) and can be quantified using a spectrophotometer at 620–655 nm.

2.3. Purification of the alginates

For purification, the 3 types of crude sodium alginate were dissolved at 4 °C in a 1 mM sodium EGTA solution to a 1% solution under constant stirring. Subsequently the solutions were filtered over successively 5.0, 1.2, 0.8, and 0.45 μm filters (Whatman®, Dassel, Germany). During this filtration step all visible aggregates were removed.

Next, the pH of the solution was lowered to 3.5 by addition of 2 N HCl + 20 mM NaCl. The solution was kept on ice to prevent hydrolysis of alginate. The next step was slow lowering of the pH from 3.5 to 2, which was associated with gradual precipitation of alginate as alginic acid [23]. Routinely, the solutions were brought at a pH of 2.0 and subsequently filtered over a Buchner funnel (pore size 1.5 mm) to wash out non-precipitated contaminants. To extend the washout of non-precipitated contaminants, the precipitate was brought in 0.01 N HCl + 20 mM NaCl, vigorously shaken, and filtered again over the Buchner funnel. This washing procedure was performed three times.

Then, proteins were removed by extraction with chloroform/butanol [24]. The alginic acid was suspended in 100 ml of 0.01 N HCl + 20 mM NaCl and supplemented with 20 ml chloroform and 5 ml 1-butanol. The mixture was vigorously shaken for 30 min and filtered over the Buchner funnel. This chloroform/butanol extraction was performed three times. Next, the alginic acid was brought in water and slowly dissolved by gradually raising the pH to 7.0 by slow addition of 0.5 N NaOH + 20 mM NaCl over a period of at least 1 h. The alginate solution obtained was subjected to a chloroform/butanol extraction to remove those proteins which can only be dissolved in chloroform/butanol at neutral pH [24]. The solution was vigorously shaken in a mixture of chloroform (20 ml at each 100 ml alginate solution) and 1-butanol (5 ml at each 100 ml alginate solution) for 30 min. The mixture was centrifuged for 3–5 min at 1800 rpm, which induced the formation of a separate chloroform/butanol phase, and was removed by aspiration. The extraction was repeated once.

The last step was precipitation of the alginate with ethanol [23]. To each 100 ml of alginate solution we added 200 ml absolute ethanol. After an incubation period of 10 min all alginate had precipitated. The alginate was filtered over the Buchner funnel and washed two times with absolute ethanol. Subsequently, the alginate was washed three times with diethyl ether. Finally, the alginate was freeze-dried overnight.

2.4. Production of microcapsules

Purified or crude alginates were dissolved at 4 °C in Krebs–Ringer–Hepes (KRH) with an appropriate osmolarity. In order to produce capsules with a similar mechanical stability we tested low-G, intermediate-G and high-G capsules from alginate solution with a different concentration (2.9, 4, and 3% respectively). For production of beads/capsules we could not apply solutions higher than the concentration indicated as this produced solutions with a viscosity above 4 cps, which is above the 0.2 μm filtration limit. This latter filtration step is required for sterilization of alginate.

The alginate solution was converted into droplets using an air-driven droplet generator as previously described [15]. Three types of beads/capsules were produced: (i) calcium-beads were formed by collecting alginate droplets in a 100 mM $CaCl_2$ (10 mM HEPES, 2 mM KCl) solution for at least 5 min. (ii) Barium-beads were formed by collecting alginate droplets in a 10 mM $BaCl_2$ solution for at least 5 min. After gelification, calcium and barium beads were washed with Krebs–Ringer–Hepes (KRH) and stored for further processing. (iii) Alginate–poly-L-lysine–alginate (APA) capsules were formed by collecting alginate droplets in 100 mM $CaCl_2$ (10 mM HEPES, 2 mM KCl) solution. After 5 to 10 min of gelification the calcium–alginate beads were suspended for 1 min in KRH containing 2.5 mM $CaCl_2$. A poly-L-lysine (PLL) membrane was formed by suspending the alginate beads in 0.1% PLL solution for 10 min. Non-bound PLL was removed by three successive washings during 3 min with calcium-free Krebs–Ringer–Hepes containing 135 mM NaCl. The outer alginate-layer was subsequently applied by 5 min incubation in ten times diluted alginate solution. The diameters of capsules and beads were measured with a dissection microscope (Leica $M77_5$, Germany) equipped with an ocular micrometer with an accuracy of 25 μm. The capsules had a diameter of 650 μm. All procedures were performed under sterile conditions [25].

2.5. Implantation and explantation of empty capsules

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 2.0 ml as assessed in a syringe with appropriate measure.

To study the tissue response we did subject the animals to laparotomy. The microcapsules were retrieved by peritoneal lavage. Peritoneal lavage was performed by infusing 5 ml KRH through a 3 mm midline incision into the peritoneal cavity and subsequent aspiration of the KRH

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