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Lipopolysaccharide quantification and alkali-based inactivation in polysaccharide preparations to enable *in vitro* immune modulatory studies

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ABSTRACT

The correct identification of immune-modulatory activity of polysaccharides is often hampered by immune-stimulatory contaminants, with pyrogens such as lipopolysaccharide (LPS) as a very potent example. In order to avoid false positive immuno-stimulatory properties to be attributed to polysaccharides, accurate quantification and inactivation of LPS in test samples is crucial. To quantify LPS in polysaccharide preparations of different origin and structure we used two different limulus amoebocyte lysate test kits in two different laboratories. We observed larger variation in detection of LPS contamination between kits than between labs. LPS quantification proved unreliable for some polysaccharide preparations as spike controls resulted in spike recoveries outside the acceptable range. We designed a cellular *in vitro* assay as alternative method to detect the presence of functional LPS. This HEK-Blue hTLR4 cell culture provides a reliable assay, when combined with a cell viability test, for determining functional LPS in polysaccharide preparations. Finally, to inactivate LPS in polysaccharide preparations, we setup an alkaline-ethanol-based treatment. With this assay we observed that our treatment (5 h incubation in 0.1 M NaOH) at 56 °C efficiently inactivated LPS in all polysaccharide preparations below immune-stimulatory levels. At this elevated temperature, however, we also observed minimal to severe degradation of polysaccharide preparations as determined with SEC-RI. Taken together, we describe methods and precautions to reliably detect and inactivate LPS in polysaccharide preparations to allow reliable *in vitro* investigations towards immune-modulatory potential of polysaccharide preparations.

1. Introduction

In recent years, polysaccharides have received increasing interest as potential immune-modulators (Schepetkin & Quinn, 2006; Wismar, Brix, Frokiaer, & Laerke, 2010). Anti-infectious (Estrada et al., 1997; Jung et al., 2004; Murphy et al., 2008; Yun et al., 1998) and anti-tumor (Harada, Itashiki, Takenawa, & Ueyama, 2010; Hong et al., 2004; Modak, Koehne, Vickers, O'Reilly, & Cheung, 2005) activities of several polysaccharides have been demonstrated in animal models. The mechanisms behind these effects are only partially understood, even though a range of polysaccharide receptors has been identified on various immune cells (Wismar et al., 2010). These carbohydrate binding receptors belong to the group of pattern recognition receptors (PRRs) that include c-type lectins, nucleotide-binding oligomerization domain (NOD)-like receptors, scavenger receptors and toll-like receptors (TLRs) (Mogensen, 2009).

In vitro and *ex vivo* studies investigating interactions between polysaccharides and immune cells can make a significant contribution to improve understanding of the diverse effects of polysaccharides on the immune system. However, the potential presence of immuno-stimulatory contaminants in polysaccharide preparations, such as microbial pyrogens, complicates this task. One of the most potent and ubiquitous immune-stimulatory contaminants is lipopolysaccharide (LPS) or endotoxin. LPS is comprised of three regions: the O-polysaccharide; the core component; and lipid A. The O-polysaccharide comprises the outer domain of LPS and consists of repeating oligosaccharide subunits that can vary greatly between bacterial strains (Lerouge et al., 2001). The core component, consisting of hetero-oligosaccharides, links the O-chain to lipid A. The biological activity of LPS is harboured in

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Lipid A (Mueller et al., 2004), which is also the most structurally conserved region of LPS (Tzeng, Datta, Kolli, Carlson, & Stephens, 2002). It consists of a phosphorylated glucosamine hetero-disaccharide acetylated with 6 fatty acid chains (Lerouge et al., 2001). Immune responses to LPS are initiated via the LPS receptor complex, which follows sub-sequential actions of LPS binding protein (LBP), CD14, lipid binding accessory protein (MD-2) and TLR-4 (Rossol et al., 2011). This complex can induce cytokine secretion by dendritic cells *in vitro* to LPS concentrations as low as 20 pg/ml (Schwarz, Schmittner, Duschl, & Horejs-Hoeck, 2014; Tynan, McNaughton, Jarnicki, Tsuji, & Lavelle, 2012).

There are many examples of false-positive immuno-stimulatory activities of plant extracts or polysaccharide preparations reported in the scientific literature that where later attributed to LPS contamination (Pugh et al., 2008; Rieder et al., 2013; Tamta et al., 2008). To obtain valuable results from *in vitro* and *ex vivo* studies regarding immuno-modulation, adequate endotoxin control is crucial (Gertsch, Viveros-Paredes, & Taylor, 2011). Activation of the clotting cascade of limulus amoebocyte lysate (LAL) from the horseshoe crab (*Limulus polyphemus*) has been the standard assay for LPS quantification for many years due to its high sensitivity (Gutsmann et al., 2010). However, the quantification of LPS is particularly difficult in polysaccharides as most methods have been developed for protein samples. In addition, LAL-based assays have also been reported to give “varying results” for protein preparations (Schwarz et al., 2014). One standard method to block LPS activity *in vitro* is the use of Polymyxin B (PmB), an antibiotic with a high binding affinity for Lipid A (Moore, Bates, & Hancock, 1986). Even though this method is convenient and effective for many samples, it is not universally applicable. In some polysaccharide preparations, PmB-mediated LPS inactivation is ineffective (Rieder et al., 2013). Interestingly, PmB is effective upon degradation of the polysaccharide sample. The ineffectiveness of PmB in certain polysaccharide preparations may therefore be due to the formation of a polysaccharide-LPS complex that hinders binding of LPS to PmB (Rieder et al., 2013). Furthermore, PmB ability to inhibit LPS-induced cytokine secretion from dendritic cells was shown to be incomplete (Tynan et al., 2012). Alternative reliable methods for LPS quantification in polysaccharide samples are therefore needed.

Here, we describe LPS quantification and inactivation in different polysaccharide preparations. We compare the LAL-based Endozyme and ToxinSensor kits in two different laboratories and demonstrate that these kits are not universally applicable for polysaccharide preparations. As an alternative, we show that HEK-Blue hTLR4 cells can reliably determine functional LPS levels. We next applied an alkaline-ethanol treatment at 4 °C and 56 °C to inactivate LPS in these polysaccharide preparations. At 56 °C we observed LPS inactivation below detection thresholds of the HEK-Blue hTLR4 assay. To monitor possible degradation of the polysaccharides, size exclusion chromatography (SEC-RI) was selected as a screening tool which revealed polysaccharide- and temperature-dependent changes in molar masses.

2. Materials and methods

2.1. Cell culture

HEK-Blue hTLR4 cells (InvivoGen, Toulouse, France) were subcultured in DMEM (Gibco, Life Technologies, Bleiswijk, The Netherlands) with 10% fetal bovine serum (FBS; Gibco) once per week and medium was refreshed twice per week.

2.2. Polysaccharide preparations

The polysaccharide preparations employed in this study originate from baker's yeast (*Saccharomyces cerevisiae*), Shiitake mushroom (*Lentinus edodes*), wheat (*Triticum aestivum*), oat (*Avena sativa*) and apple (*Malus domestica*). Water soluble 1,3/1,6 beta-glucan from yeast (Wellmune Soluble) was provided by Biothera (Eagan, MN, USA) and contained $>90\%$ β-glucan (according to the manufacturer). Linear, mixed linkage β-1,3/1,4-glucan from oat with a purity of $>90\%$ (OBG90) was provided by Swedish Oat Fiber (Bua, Sweden). A water soluble, arabinoxylan-enriched, fraction from wheat (Naxus) (55% arabinoxylan based on sum of arabinose and xylose) was provided by Bioactor (Maastricht, The Netherlands). A rhamnogalacturonan-I fraction from apple pectin (RG-1) (76% pectin based on the sum of rhamnose, arabinose, galactose and uronic acid) was provided by INRA (INRA, Nantes, France). Lentinan containing extract from shiitake (LCES; 51% β-glucan based on starch-free glucose content) was acquired based on a previously described method (Tomassen, Hendrix, Sonnenberg, Wichers, & Mes, 2011). In brief, 660 gr fresh shiitake was homogenized in a magimix blender. The grounded pieces were added to 1 l of boiling tap water. The samples were further homogenized with a blender and boiled for 8 h. After O/N cooling the extracts were centrifuged at 2800 rpm for 30 min. The supernatant was collected and precipitated O/N by addition of 1 vol of 96% ethanol. The precipitate was collected and lyophilized. The constituent sugar content used for purity estimation is based on alditol acetate derivatives quantified by GC after acidic hydrolysis and derivatization (Blakeney, Harris, Henry, & Stone, 1983).

2.3. Preparation of polysaccharide samples for LPS quantification and cell culture experiments

At lab 1, the polysaccharide samples were dissolved in endotoxin free water (<0.005 EU/ml, G-Biosciences, MO, USA) at a concentration of 1 mg/ml in endotoxin free centrifuge tubes (<0.05 EU/ml following water washing of tubes) by incubation in a boiling water bath for 1 h. Prior to LPS quantification by the LAL method, samples were diluted stepwise (1:10) with endotoxin free water. At lab 2, the polysaccharide samples were dispersed in endotoxin free water (Life Technologies-Gibco, Bleiswijk, The Netherlands) in endotoxin-free glass vials (Hyglos, Bernried am Stranberger See, Germany) at a concentration of 3 mg/ml and stirred for 6 h and stored overnight (O/N) at ambient temperature (RT). Subsequently, samples were diluted with endotoxin free water to 0.75 or 1 μg/ml for quantification of LPS by the LAL method. For use in cell culture experiments, polysaccharides were dispersed O/N in appropriate endotoxin free culture medium at 40 °C.

2.4. LPS quantification

2.4.1. LPS quantification with commercial LAL based test kits

LPS quantification in the different polysaccharide samples (prepared as described above) was conducted with two different commercial test kits. Both test kits did not contain the specific recognition protein for β-glucan (factor G). ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit was purchased from GenScript (GenScript, NJ, USA). EndoZyme® test kit, which contains recombinant factor C, was purchased from Hyglos (Hyglos GmbH, Bernried am Starnberger See, Germany). Both test kits were used according to the manufacturer's instructions. For the EndoZyme® test kit, this instruction included the use of spike controls alongside all tested polysaccharides. Linear regression models were used for both kits to construct the calibration curves based on log concentration and log fluorescence or log absorption readings at both labs. For the Endozyme test kit, LPS standards in a range from 0.005 to 5 EU/ml were used. In comparison, the ToxinSensor™ test kit can be used either in the range of 0.01-0.1 or in the range of 0.1–1 EU/ml. At both labs, spiking was performed by adding LPS to