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Investigation of the biodistribution, breakdown and excretion of delta inulin adjuvant

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ABSTRACT

Insoluble, nanostructured delta inulin particles enhance the immunogenicity of co-administered protein antigens and consequently are used as a vaccine adjuvant (Advax™). To better understand their immunomodulatory properties, the *in vitro* hydrolysis and *in vivo* distribution of delta inulin particles were investigated. Delta inulin particle hydrolysis under bio-relevant acidic conditions resulted in no observable change to the bulk morphology using SEM, and HPLC results showed that only 6.1% of the inulin was hydrolysed over 21 days. However, 65% of the terminal glucose groups were released, showing that acid hydrolysis relatively rapidly releases surface bound chemistries. This was used to explain *in vivo* biodistribution results in which delta inulin particles surface-labelled with fluorescein-5-thiosemicabizide were administered to mice using intramuscular (I.M.) or subcutaneous (S.C.) routes. Comparison analysis of the fluorescence of soluble inulin in the supernatants of homogenised tissues maintained at room temperature or heated to 100 °C to solubilise particulate inulin was used to distinguish between fluorescent probe on soluble inulin and probe bound to inulin within particles. Following both I.M. and S.C. injection delta inulin exhibited a depot behaviour with local injection site residence for several weeks. Over this time, as injection site inulin reduced, there was measurable transport of intact delta inulin particles by macrophages to secondary lymphoid organs and the liver. Ultimately, the injected delta inulin became solubilised resulting in its detection in the plasma and in the urine. Thus injected delta inulin particles are initially taken up by macrophages at the site of injection, trafficked to secondary lymphoid tissue and the liver, and hydrolysed resulting in their becoming soluble and diffusing into the blood stream, from whence they are glomerularly filtered and excreted into the urine. These results provide important insights into the biodistribution of I.M. or S.C. injected delta inulin particles when used as vaccine adjuvants and their method of excretion.

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1. Introduction

Inulin is a plant-based fructan comprised of a linear chain of fructose units connected by β-(2–1) glycosidic bonds and capped at the reducing end by an α-D-(1–2)-glucopyranoside ring. Inulin was established as non-toxic and safe for human use by US Food and Drug Administration in 1992 (GRAS Notice No. GRN 000118) and is used clinically for kidney function testing due to the tropism of soluble, short chain inulin to the kidneys followed by rapid excretion [1]. Longer chain inulin can be precipitated into specific semicrystalline forms made up of inulin chains that aggregate together into antiparallel helices through hydrogen bonding to form lamellar sheets [2]. These 'isoforms' increase in melting and dissolution temperature as defined by the increasing number of inulin helical unit cells contributing to the crystalline layers [3]. Semicrystalline forms of inulin insoluble at 37 °C, such as delta inulin or Advax™, prevent entry into the circulation when injected peripherally. This prevents rapid renal excretion, increasing the biological half-life of the inulin particles and allowing their binding, uptake and transport by monocytes [1]. This interaction with the immune system results in enhancement of humoral and cellular immune responses to a wide variety of co-administered viral

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[4–9] bacterial [10–17] and protozoan antigens as well as toxins and allergens [18] demonstrating utility as a vaccine adjuvant across a broad range of animal models, as well as in humans [19]. Surprisingly for a vaccine adjuvant, delta inulin does not activate classical inflammatory innate immune receptors such as the toll like receptors, but instead works uniquely by directly modulating the function of dendritic cells, enhancing antigen presentation to memory T and B cells [20]. Consequently, delta inulin operates by a non-inflammatory mode of adjuvant action, helping to explain its very low reactogenicity. This makes it of great interest for use in modern vaccines based on recombinant proteins and peptides that otherwise lack immunogenicity. The immunomodulatory properties of inulin particles has also found benefit in anticancer treatments [21,22] and provides opportunities for enhancing drug responses, while the tropism to monocytes presents opportunities for targeted drug delivery. The increasing use of inulin in pharmaceuticals such as drug delivery vehicles [23–30] and the increasing data surrounding delta inulin as a vaccine adjuvant means that greater understanding of its *in vivo* behaviour is required. Consequently, this present study characterises the *in vitro* hydrolysis and *in vivo* biodistribution of delta inulin to help explain its intriguing and largely unexplained biological properties and its unique interaction with the immune system. The results provide a better understanding of immunomodulatory effect of delta inulin and this knowledge will be important in the continuing development of delta inulin as a vaccine adjuvant as well as for its potential use in other pharmaceutical products and delivery systems.

2. Materials and methods

2.1. Materials

Delta inulin was prepared by Vaxine Pty Ltd, Adelaide, Australia, as described previously [31]. Sodium meta periodate, sodium acetate, acetic acid, glycerol, ethanolamine, acetonitrile, sodium cyanoborohydride solution (5 M in 1 M NaOH) and fluorescein-5-thiosemicabizide were all purchased from Sigma–Aldrich and phosphate buffered saline (PBS, pH 7.4, 0.01 M) solutions were prepared from Sigma–Aldrich tablets using pure water. Components used to prepare Artificial Lysosomal Fluid (ALF) (Table S1, supporting information) were all sourced from Sigma-Aldrich. DMSO and Black 96 well plates were purchased from Thermo Fisher Scientific. Saline solutions were purchased from InterPharma (sodium chloride for injection BP 0.9%). All solutions were prepared from pure water obtained from a MilliQ water purification system operating at a resistance of 18.2 MΩ/cm.

2.2. Methods

2.2.1. In vitro delta inulin hydrolysis in artificial lysosomal fluid (ALF)

2.2.1.1. Preparation of ALF. ALF was prepared in accordance with previous studies [32]. The following materials in Table S1 (supporting information) were combined with MilliQ water and mixed until completely dissolved for the preparation of ALF at pH 4.5.

2.2.1.2. In vitro hydrolysis of delta inulin particles. Delta inulin particles were dispersed in 15 mL of ALF at a concentration of 10 mg/mL. The cleavage media was kept at 37 °C and stirred continuously at 200 Revolutions per minute (rpm). 500 μL of aliquots were periodically taken and were centrifuged immediately. The supernatant was neutralized by addition of 60 μL saturated sodium bicarbonate solution to quench further acid-mediated hydrolysis. Both the supernatant and pellet were retained for analysis.

The concentration of fructose, glucose and sucrose cleaved from inulin particles was analysed using a HPLC system (Shimadzu Corporation, Japan) consisting of a series of LC-20ADXR pumps, SIL-20ACXR auto sampler, CTO-20AC column oven set at 30 °C, and ELSD-LTII evaporative light scattering detector, and a Luna amino analytical column (NH_2, 5 μm, 4.6 mm ID × 250 mm). The mobile phase was a mixture of acetonitrile and MilliQ water (95:5 v/v), eluted at a flow rate of 1.0 ml/min. The limit of detection (LOD) of the analytical method was 20 μg/ml for all sugars. Linear calibration curves ($R^2 \geq 0.99$) were plotted for chromatographic peak areas against sugar concentrations over the range of 33–1000 μg/ml, without the addition of an internal standard. All analytes were diluted suitably to meet the calibration concentration range.

The pellet was washed three times in MilliQ via redispersion followed by centrifugation at relative centrifuge force (rcf) of 4500 for 90 s. The particle size, structure and surface morphology of inulin particles were examined by high-resolution analytical scanning electron microscopy (Zeiss Merlin, Oberkochen, Germany). Inulin particles were dispersed in MilliQ at a concentration of 0.1 mg/mL and a 5 μL sample was placed on a silicon wafer prior to sputter coating with 5–10 nm gold. Imaging was performed at an accelerating voltage of 1 kV.

^{1}H NMR spectroscopy was used to measure the chain length of inulin particles using the method published previously [33]. Briefly, ^{1}H NMR spectra were recorded on a Bruker Avance III 600 at 600 MHz for ^{1}H. The integral of the glucose anomeric peak at 5.44 ppm was set to 1 to calibrate a spectrum and the combined integral of all other resonances of inulin (=X) provided a measure of the average number of units in the chain using the formula: DPn = ((X − 6)/7) + 1. Duplicate ^{1}H NMR spectra were each analysed three times to give an overall average for DPn that proved reproducible, with CV of replicate assays generally ~0.5%. In this case, the delta inulin had an average inulin chain length of 40.1 ± 1.1 sugar units.

2.2.2. Fluorescent modification of delta inulin particles

10 mg/ml sodium meta periodate was dissolved in sodium acetate buffer (SAB, 0.1 M, pH 5.5) in a light-protected container. 45 mg inulin particles were dispersed in another light protected container containing 3.52 ml SAB buffer. Then 0.98 ml of periodate solution was added to the solution. The mixture was then gently agitated for 30 min at room temperature before the reaction was quenched by the addition of 0.45 ml glycerol. The particles were then centrifuged at 3000 rcf and the supernatant discarded followed by washing three times with phosphate buffered saline (PBS) (4500 rcf). The oxidised inulin particles were redispersed into 4.5 ml PBS. To this was added fluorescein-5-thiosemicabizide and the mixture was agitated gently at room temperature for 2 h. 45 μl sodium cyanoborohydride solution was then added and the reaction continued for a further 30 min before quenching with 450 μl ethanolamine solution (1 M) for 1 h. The delta inulin particles were then washed by centrifuging at 4500 rcf and replacing the supernatant with PBS until the supernatant lost the orange colour.

2.2.3. Animal work

Delta inulin *in vivo* biodistribution was investigated by two different administration routes, namely I.M. and S.C. Adult BALB/c 6–8 weeks of age (weighting an average of 22.8 g) were purchased from Flinders University School of Medicine animal facility. Mice were bred and maintained under specific-pathogen-free conditions, and the animal ethics No. 830/12 was approved by the Animal Welfare Committee of Flinders University School of Medicine. Mice were fed with standard diet and allowed water. All experiments were carried out in compliance with animal management roles. Adult BALB/c mice were administered 4 mg of fluorescently labelled delta inulin dispersed in PBS (100 μl) by an I.M. or S.C. injection in one hind limb. Subsequently, urine samples from 3

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