



HHS Public Access

Author manuscript

Vaccine. Author manuscript; available in PMC 2016 November 04.

Published in final edited form as:

Vaccine. 2015 November 4; 33(44): 5920–5926. doi:10.1016/j.vaccine.2015.09.030.

Advax™, a novel microcrystalline polysaccharide particle engineered from delta inulin, provides robust adjuvant potency together with tolerability and safety

Nikolai Petrovsky^{1,2} and Peter D. Cooper^{1,3}

¹Vaxine Pty Ltd, Flinders Medical Centre, Adelaide, SA, Australia, 5042

²Department of Endocrinology, Flinders Medical Centre and Flinders University, Adelaide, Australia 5042

³John Curtin School of Medical Research, Australian National University, Canberra, Australia 2061.

Abstract

There is an ongoing need for new adjuvants to facilitate development of vaccines against HIV, tuberculosis, malaria and cancer, amongst many others. Unfortunately, the most potent adjuvants are often associated with toxicity and safety issues. Inulin, a plant-derived polysaccharide, has no immunological activity in its native soluble form but when crystallised into stable microparticles (delta inulin) acquires potent adjuvant activity. Delta inulin has been shown to enhance humoral and cellular immune responses against a broad range of co-administered viral, bacterial, parasitic and toxin antigens. Inulin normally crystallises as large heterogeneous particles with a broad size distribution and variable solubility temperatures. To ensure reproducible delta inulin particles with a consistent size distribution and temperature of solubility, a current Good Manufacturing Practice (cGMP) process was designed to produce Advax™ adjuvant. In its cGMP form, Advax™ adjuvant has proved successful in human trials of vaccines against seasonal and pandemic influenza, hepatitis B and insect sting anaphylaxis, enhancing antibody and T-cell responses while at the same time being safe and well tolerated. Advax™ adjuvant thereby represents a novel human adjuvant with positive effects on both humoral and cellular immunity. This review describes the discovery and development of Advax™ adjuvant and research into its unique mechanism of action.

Keywords

adjuvant; vaccine; delta inulin; Advax; immunity

Corresponding author: Prof. Nikolai Petrovsky, Endocrinology Dept, Flinders Medical Centre, Bedford Park, SA 5042, AUSTRALIA, nikolai.petrovsky@flinders.edu.au, Ph +61 8 8204 4572, Fax +61 8 8204 5987.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Inulin: historical background

Inulin is a simple plant-based fructan polysaccharide comprising a family of linear $\beta(2\rightarrow1)$ polyfructofuranosyl α -D-glucose polymer chains in which an unbranched chain of fructose rings is terminated with a single glucose (Fig. 1). Inulin utilised as a storage carbohydrate by plants of the *Compositae* family that include dahlias, chicory, artichoke, onions, and garlic (reviewed in [1]). Inulin's medicinal uses date back to ancient times. Pedanios Dioscoride, a physician with the Roman army, in 100AD reported the beneficial effects of chicory root extract (~40% inulin by weight) for treatment of stomach, liver and kidney complaints [2]. In 1804, inulin was extracted by a German scientist from a boiling water extract of *Inula helenium* [3]. Based on its *Inula* source, Thomson in 1818 gave this extract the name, inulin [4]. Oral inulin's beneficial effects on metabolic disorders was re-discovered in 1874 with a report that diabetic patients lost their glycosuria when put on a diet of 100 grams of inulin per day [3]. Dietary inulin not only has a favourable effect on blood sugar levels in patients with type 2 diabetes but also reduces fasting insulin concentrations, triacylglycerol levels [5] and hepatic lipogenesis [6]. Inulin has also been shown to reduce atherosclerotic lesions in Apo E-deficient mice [7]. Furthermore, short chain fatty acids including butyrate and propionate formed by inulin fermentation in the gut have anti-proliferative effects [8] and stimulate apoptosis of colon cancer cells, enhancing expression of enzymes including histone deacetylases involved in detoxification of carcinogens [9]. Inulin fermentation products in the gut also have anti-inflammatory effects and may help suppress autoimmune disease [10].

Use of inulin for measurement of human glomerular filtration

Although inulin can be metabolised by gut flora, the inability of mammals to metabolise injected inulin led Shannon and Smith in the 1920's to explore inulin's use for measurement of glomerular filtration (GFR) [11]. They confirmed the nontoxicity of inulin by intravenously injecting themselves with 160 grams of soluble inulin thereby confirming inulin's exceptional safety [11]. Injection of soluble inulin subsequently became the gold standard for GFR measurement, with intravenous injection of a loading dose of 50 mg/kg body weight followed by an infusion to maintain steady plasma levels. Measurement of urinary inulin then provided an estimate of GFR. Many thousands of patients have safely had this GFR test including pregnant women and newborn babies with no known adverse effects attributable to inulin other than a mild osmotic diuresis [12]. In the early days hospitals made their own inulin formulations and patients receiving inulin infusions occasionally developed transient hypotensive symptoms. This was found to reflect complement activation that was independent of the only then known (now called classical) complement pathway. A search for the mechanism of complement activation by inulin led to discovery of the alternative complement pathway (ACP) [13]. ACP activation was found to occur when injected inulin was not entirely dissolved, with microscopic crystalline inulin contaminants responsible for the ACP activation. Hence it was found that ACP activation is a unique feature of crystalline but not soluble inulin, a feature critical to its later use as a vaccine adjuvant as described below.

Development of gamma inulin as an anti-cancer agent

Complement activation has been shown to be effective in killing cancerous cells in animals and humans. An early demonstration of this anti-cancer effect of complement utilised the complement activator, *Staphylococcus aureus* protein A, albeit with major side effects including pyrexia, nausea, vomiting, and significant cardiopulmonary toxicity attributed to enterotoxin B contamination and formation of immune complexes [14]. To avoid the problems of using *S. aureus* for complement activation, an alternative approach to cancer therapy was developed by Cooper et al. with the idea of using inulin crystals instead for complement activation [15]. This work led to the discovery of a crystalline isoform of inulin which was given the name gamma inulin, to distinguish it from the previously known and highly soluble alpha and beta inulin isoforms [16]. Gamma inulin was potent at ACP activation in human plasma reaching a maximum at ~20µg/ml [17]. In mice, intraperitoneal administration of gamma inulin at doses of 0.2 - 0.5 mg induced greater than 50% serum complement depletion within 30 minutes with complement levels returning to normal within 16 - 24 hours [18]. Despite the potent ACP activation, intraperitoneal or subcutaneous injections of gamma inulin in mice at doses up to 10 mg were well tolerated with mice not exhibiting any adverse effects. Intraperitoneal administration of gamma inulin to mice implanted with a B16 melanoma cell line prolonged survival times, whereas depletion of complement factor 3 (C3) in the mice abrogated this protective effect, consistent with gamma inulin's anti-tumour effects being mediated by ACP activation [19]. Gamma inulin similarly induced tumour regression when injected into squamous cell carcinomas in sheep, an effect enhanced by combination therapy with cyclophosphamide [18]. Intralesional injection of gamma inulin into equine sarcoids or spontaneous tumours in dogs similarly resulted in tumour regression (P. Cooper, personal communication). Gamma inulin also potentiated anti-cancer effects of photodynamic therapy in an ACP-dependent manner [20].

Development of microcrystalline inulin as an immune adjuvant

Complement as a key component of the innate immune system also plays an important role in generation of adaptive immunity. This effect of complement has been exploited in vaccine design and, for example, covalent coupling of antigens to complement factor C3d was shown to enhance their immunogenicity [21]. Given its potent ability to activate complement, we asked whether co-formulation of antigens with gamma inulin might similarly enhance their immunogenicity. When tested in this way, gamma inulin proved to have a beneficial effect on adaptive immune responses. Many of the early studies were performed with keyhole limpet hemocyanin (KLH) and when KLH was co-administered with gamma inulin this resulted in enhanced anti-KLH antibody responses in mice with similar results later obtained in guinea pigs and rabbits [22] with gamma inulin subsequently shown to improve the immunogenicity of a range of vaccine antigens (reviewed in [23]).

The mechanism of adjuvant action of gamma inulin was shown to involve increased C3 deposition on the surface of macrophages leading to enhanced T-cell activation [24]. Although it did not induce macrophage activation as measured by chemiluminescent respiratory burst, gamma inulin primed macrophages for a greater respiratory burst in response to phorbol ester. Incubation of human peripheral blood mononuclear cells with

tetanus toxoid in the presence of gamma inulin resulted in enhanced IL-2 secretion when compared to toxoid alone, consistent with augmented antigen processing and presentation [18]. Cobra venom depletion of serum complement in mice prior to immunization blocked gamma inulin's adjuvant effects, consistent with adjuvant activity being dependent upon ACP activation [24]. In an attempt to enhance its adjuvant potency gamma inulin was subsequently co-crystallised with aluminium hydroxide to form Algammulin, which proved more potent than gamma inulin in enhancing vaccine antibody responses [17]. Nevertheless, Algammulin had significant drawbacks that prevented its development as a human vaccine adjuvant including ongoing concerns regarding potential toxicity of aluminum adjuvants [25, 26], potential issues associated with an excessive Th2-immune bias [27, 28], and high batch-to-batch variability in its adjuvant potency. To overcome these problems, studies were undertaken into the structure and function of inulin with the aim of developing a more stable and consistent inulin adjuvant platform. This led to development of the delta inulin isoform that not only had much greater and more consistent adjuvant activity than gamma inulin but most importantly was able to be manufactured as a highly consistent cGMP product [29]. Delta inulin subsequently formed the basis of the Advax™ adjuvant platform.

Structural basis of delta inulin

Inulin has a hydrophobic polyoxyethylene-like backbone that is critical to its structure in solution and when crystallised. Inulin particles obtained by precipitation from cold water are referred to as alpha inulin and the particles obtained by precipitation from ethanol as beta inulin; dried commercial inulin is almost all in alpha or beta forms. Both alpha and beta isoforms are highly soluble and readily dissolved in water at 25°C, gamma inulin starts to become soluble in water at 37°C, and delta inulin is insoluble at temperatures below 40°C, an important distinction as this means that delta inulin particles are insoluble at body temperatures. Hence, the key factor behind understanding of delta inulin is the fact that inulin polymers can be crystallised into discrete isoforms characterised by different solubility rates in aqueous media [29, 30]. In essence, each inulin particle is made up of individual inulin polymers each formed into an antiparallel double helix, with these helices then being aggregated together through lateral hydrogen bonding to form lamellar sheets [29, 31, 32]. The greater stability of delta inulin when compared to earlier inulin isoforms thereby reflects greater aggregate strength of H-bonding between each of the individual inulin polymers forming the crystal structure, with the average length of the polymers making up the isoforms increasing with progression up the polymorphic series [30]. Transmission and scanning electron microscopy and atomic force microscopy studies of delta inulin particles show consistent spherulite-like discoid particles 1-2 microns in diameter made up of a series of lamellar sheets [33]. Better knowledge of the structural basis of delta inulin crystallization enabled design of a cGMP production process, thereby ensuring ability to produce Advax™ adjuvant particles with a highly consistent size and reproducible adjuvant properties, as shown in Figure 2, in marked contrast to the highly irregular shape, size and inconsistent adjuvant properties of gamma inulin and initial delta inulin formulations.

Alternative complement pathway activation

Gamma and delta inulin both activate the complement via the ACP [29]. It is not known exactly why these inulin structures are such potent complement activators, although they do have a high number of surface hydroxyl groups that are recognised as important to complement activation. Virtually any biological surface can activate the ACP provided no sialic acid moieties are present, as sialic acid functions as a discriminator to protect vertebrate surfaces from self-complement attack [34]. Complement factor C3b has only a moderate preference for spontaneous reaction with glucose or fructose moieties [35]. Hence, inulin polymers themselves have no particular features to suggest high C3 convertase activation activity. Indeed, soluble inulin polymers have no ability to activate complement explaining their safety in human renal function testing. For ACP initiation to lead to the rapid amplification of complement activation, the labile C3 convertase products produced by ACP activation must be localized on a surface site relatively protected from circulating complement inhibitors and degrading enzymes [35]. Therefore, it is possible that the surface of gamma and delta inulin crystal packing structures is most relevant to their ability to activate complement. Despite potent ACP activation capability, both gamma and delta inulin are extremely well tolerated even when injected at high doses. This suggests that the complement activation induced by these inulin isoforms occurs in a controlled fashion that avoids excess release of toxic downstream mediators. We hypothesize that delta inulin activates complement regulators such as clusterin thereby blocking production of downstream anaphylatoxins and complement membrane attack complexes [36]. If so, this could explain how delta inulin is able to separate the beneficial effects on adaptive immunity of upstream complement activation factors such as C3d, from the harmful downstream effects on cell integrity of complement membrane attack complexes.

Preclinical vaccine studies

Delta inulin has multiple advantages as an adjuvant over gamma inulin including greater temperature stability, ability to be produced as a monodisperse particle population under cGMP (Fig. 2) and improved adjuvant potency in animal models. Delta inulin is also resistant to damage from sterilizing doses of gamma irradiation to which gamma inulin is highly susceptible [37]. Consequently, development of gamma inulin ceased more than a decade ago with efforts instead being refocused on delta inulin adjuvant development, in the form of Advax™ adjuvant. An important distinction is that whilst Advax™ adjuvant is comprised of delta inulin, the terms Advax™ and delta inulin are not synonymous, with Advax™ adjuvant also comprising delta inulin of highly specific particle sizes and morphology, thereby distinguishing it from delta inulin more generally.

Early studies showed that immunization of mice with hepatitis B surface antigen (HBsAg) plus Advax™ adjuvant provided at least 4-fold antigen-dose sparing when compared to a standard alum-adjuvanted vaccine [29]. The Advax™-immunized mice also showed a more balanced Th1 and Th2 immune response by contrast to the extreme Th2-bias seen in mice immunized with alum adjuvant. Use of Advax™ adjuvant was associated with increased CD4⁺ and CD8⁺ T-cell proliferative responses and increased antigen-stimulated production of IFN γ , TNF α , IL-4, IL-5, IL-6, IL-10, IL-13, IL-17 and GM-CSF [38], consistent with

broad based enhancement of all T-cell subsets, rather than the strong Th2- or Th1-bias of alum or CpG adjuvants, respectively [39]. IL-1 α production was not increased by AdvaxTM adjuvant [38] suggesting that unlike alum [40], AdvaxTM does not induce inflammasome activation. Its adjuvant effect was not dependent on antigen absorption as antibody responses were enhanced even when AdvaxTM was injected one day prior to the antigen [38].

Advax's effects on influenza vaccines have been extensively studied. Formulation of inactivated influenza vaccine with AdvaxTM enhanced serum neutralizing antibody titers in mice and provided up to 100-fold antigen sparing [41]. This correlated with a higher frequency of influenza-specific IgM- and IgG-secreting B cells in the bone marrow and spleen of immunized mice. As seen for HBsAg immunization, use of AdvaxTM was associated with an increase in influenza-specific CD4+ and CD8+ T-cell proliferation and increased IFN- γ , IL-2, IL-5, IL-6, and GM-CSF production, which translated into enhanced protection against lethal influenza challenge [41]. Immunity was long-lived with high antibody titers, T-cell responses and influenza protection still evident 1 year post-immunization with AdvaxTM. Injection of mice with AdvaxTM alone in the absence of antigen provided no protection against influenza infection, indicating that it does not induce non-specific innate immune protection. AdvaxTM has also been shown to boost the immunogenicity of inactivated influenza vaccine even when delivered via the intrapulmonary route [42]. AdvaxTM adjuvant was also tested for its ability to help overcome the effects of pregnancy-associated immune suppression on influenza vaccine responses. AdvaxTM adjuvant was well tolerated by pregnant dams and was not associated with any adverse reproductive or developmental effects in mothers or pups [43]. Pregnant dams that received a single intramuscular injection of inactivated influenza antigen with AdvaxTM adjuvant had increased serum anti-influenza IgG titers and this translated into higher anti-influenza IgG titers in their breast milk. This in turn resulted in higher anti-influenza IgG titers in the serum of their suckling pups and these pups were completely protected when challenged with influenza at 4 weeks-of-age. By contrast, no survival was seen in pups of mothers immunized with a standard unadjuvanted influenza vaccine [43]. In a ferret challenge model, AdvaxTM adjuvant enhanced protection conferred by a licensed vaccine against high-pathogenicity avian (H5N1) influenza. Two immunizations with inactivated H5N1 antigen plus AdvaxTM adjuvant with or without the addition of CpG oligonucleotide induced high serum H5N1 neutralizing antibody titers whereas antibody was barely detectable in ferrets that received standard vaccine [44]. Enhanced survival, reduced clinical disease and absence of brain invasion by virus was seen in the AdvaxTM-adjuvanted groups with the virus being completely cleared from the nasal wash by day 4 post-challenge.

AdvaxTM adjuvant was similarly shown to enhance vaccine protection against pulmonary anthrax. AdvaxTM enhanced antibody responses to recombinant protective antigen (rPA) resulting in enhanced protection against a lethal anthrax challenge even after just a single immunisation [45]. A synergistic effect was seen when murabutide, a NOD2 agonist, was co-formulated with the AdvaxTM adjuvant. As shown by *in vivo* imaging of cathepsin cleavage of ProSense 750, AdvaxTM and murabutide induced significantly less local injection site inflammation than the alum adjuvant.

Flaviviruses represent another major vaccine challenge. Although there are human vaccines against Japanese encephalitis virus (JEV) [46], there are currently no licensed vaccines against West Nile virus or dengue. Inclusion of Advax™ adjuvant in an inactivated JEV antigen not only enhanced neutralising antibody responses in immunized mice and horses but was also associated with induction of cross-neutralizing antibody responses against Murray Valley encephalitis virus (MVEV) and West Nile virus (WNV) [47]. It was subsequently demonstrated that the Advax™ adjuvant enhanced production of memory B cells able to transfer protection against JEV and related flaviviruses including MVEV to naïve mice [48]. Cross-protection against WNV was achieved after just a single dose of JEV vaccine formulated with Advax™ adjuvant and was maintained out to at least one year post-immunization [49]. This same Advax™-adjuvanted JEV vaccine was found to be safe and effective when administered to pregnant horses and newborn foals in which it similarly induced broadly cross-protective anti-flavivirus antibodies [50].

Advax™ adjuvant enhanced neutralizing antibody responses and protection in a murine severe acute respiratory syndrome (SARS) coronavirus vaccine model. Formulations of inactivated SARS virus or recombinant spike protein with Advax™ adjuvant protected mice and reduced virus titers in the lung when compared to immunization with either antigen alone [51]. One year post-immunization, animals immunized with Advax™-adjuvanted vaccine had higher SARS-specific CD4+ and CD8+ T-cell proliferation responses and IFN γ , IL2, IL4, IL6, IL10, IL17 and TNF α -production. Whereas alum-formulated SARS antigens were associated with increased eosinophilic lung immunopathology in response to challenge with SARS virus, lung immunopathology was absent in mice immunized with delta inulin-adjuvanted vaccine. The absence of lung immunopathology in the Advax™ adjuvant groups correlated with a higher frequency of SARS-specific IFN α -producing T cells, consistent with Advax™ adjuvant driving a more balanced Th1 and Th2 response. Inhibition of eosinophilic lung immunopathology was further assisted by co-formulation of Advax™ adjuvant with CpG oligonucleotide [51].

Advax™ adjuvant alone or in combination with CpG oligonucleotide enhanced the immunogenicity in mice of an HIV envelope protein boost following a DNA plasmid intramuscular prime, thereby inducing persistent mucosal gp120-specific IgA, systemic IgG and memory T- and B-cell responses [52]. Similarly, in rabbits and nonhuman primates Advax™ adjuvant enhanced production of simian immuno-deficiency virus cross-neutralising antibodies when combined with a envelope protein boost that followed a DNA vaccine prime [53].

Advax™ adjuvant enhanced protection afforded by a *Listeria* vaccine based on T-cell peptide epitopes conjugated to gold nanoparticles [54, 55]. Enhanced protection by the Advax™ adjuvant correlated with an increased frequency of splenic CD4+ and CD8+ T cells, NK cells, CD8+ DC and Th1 cytokine production (IL12, IFN γ , TNF α and MCP1) and was associated with increased T-cell epitope spreading following live *Listeria* challenge [56].

Examples of veterinary vaccines where Advax™ adjuvant has been shown to have beneficial effects on neutralizing antibody responses and/or protection include in vaccines

against *Peste de petit ruminants* in mice and goats [57] and against African Horse Sickness and Meliodosis in camels [58].

Administration routes, species-specificity and mechanism of action

In most of the above-described preclinical studies, Advax™ adjuvant was administered intramuscularly or subcutaneously to mimic its intended human use. However, Advax™ adjuvant is also effective if given via other routes, including intradermal, intraperitoneal or intranasal delivery (unpublished data). It was recently shown to have adjuvant activity even when administered directly into the murine lung. Intratracheal administration of a dry powder inactivated influenza vaccine together with Advax™ adjuvant induced higher influenza-specific intranasal IgA titers and a more balanced Th1/Th2 antibody isotype response with no observed adverse effects [42].

Whilst the vast majority of immunogenicity studies of Advax™ adjuvant have been undertaken in mice, adjuvant activity has been demonstrated across a broad range of other animal species including guinea pigs [38], ferrets [44], goats [57], horses [47] and camels [58]. To date, no species limitation in Advax™ adjuvant action has been seen with adjuvant activity also seen in rats, rabbits, dogs, cats and sheep (unpublished data). It is also effective across different life stages, being efficacious and safe when administered in combination with inactivated influenza vaccine to pregnant mice [43] or 7-day-old mouse pups [59] or in combination with inactivated Japanese encephalitis vaccine to pregnant mares or newborn foals [50].

Most adjuvants have been shown to work via danger signals. For example, aluminium salts inducing cell necrosis resulting in inflammasome activation [60] and also triggering toll-like receptor (TLR)-9 activation through DNA released from lysed cells [61]. Similarly, monophosphoryl lipid A activates TLR4, flagellin activates TLR5 and CpG oligonucleotides activate TLR9. The final common pathways for all these adjuvants working via danger signals is activation of the transcription factor, nuclear factor-kappa B (NFκB), the master regulator of inflammatory responses [62]. Advax™ adjuvant is an exception as it fails to induce NFκB activation in human monocytes or murine splenocytes and similarly does not activate the inflammasome or induce inflammatory cytokine production (unpublished data). The mechanism underlying the unique ability of Advax™ adjuvant to separate innate immune activation and inflammation from enhancement of adaptive immunity is the subject of intense ongoing study by our group.

Clinical Development

A first-in-man Phase 1 clinical trial was conducted to assess the safety and tolerability of three intramuscular doses of HBsAg formulated with Advax™ adjuvant in healthy adult subjects was undertaken following successful preclinical acute and repeat dose toxicology studies in which no safety problems were identified [63]. Advax™ adjuvant was well tolerated by the trial subjects with injection site pain scores not significantly different to subjects receiving HBsAg alone. Advax™ enhanced anti-HBsAg antibody titers and seroprotection rates when compared to administration of HBsAg alone. The proportion of

subjects with positive anti-HBsAg CD4+ T-cell responses was also significantly higher in subjects that received Advax™ adjuvant [63].

A Phase 1/2 study was undertaken in adult subjects to assess the ability of Advax™ adjuvant to enhance the immunogenicity of a pandemic influenza A/H1N1/2009 pdm vaccine made from recombinant hemagglutinin [64]. Advax™ increased seroprotection rates by 1.9 times after the first, and 2.5 times after the second, immunization when compared to immunization with the recombinant hemagglutinin alone. Use of Advax™ adjuvant was tolerated with no adjuvant-associated adverse reactions observed.

Advax™ adjuvant has also been found to be safe and effective when combined with a bee venom-based immunotherapy administered to human subjects with bee-sting anaphylaxis with accelerated and higher titers of venom-specific IgG4, a marker of successful immunotherapy [65]. Other clinical trials where Advax adjuvant has been successfully tested include in combination with seasonal trivalent inactivated influenza vaccines and in combination with a universal T-cell vaccine against influenza based on synthetic peptide epitopes (unpublished data).

Conclusions and Future Prospects

Advax™ adjuvant based on delta inulin microcrystalline particles successfully enhanced vaccine immunogenicity across a broad range of antigen types including whole inactivated viruses, recombinant proteins, synthetic peptides, toxins and venoms. It has been effective across all animal species tested so far, active across a variety of immunization routes and effective when given during pregnancy or in early neonatal life. It consistently enhances serum antibody titers and CD4+ and CD8+ T-cell immunity to co-administered antigens and acts synergistically with traditional innate immune activators such as murabutide or CpG oligonucleotides. Early promise in preclinical studies has been supported by successful human clinical trials confirming its safety, tolerability and efficacy. Ongoing priorities are to further characterise delta inulin's mechanism of action, develop it as a mucosal adjuvant, explore synergistic combinations with other immune activators and extend human clinical studies to paediatric populations.

ACKNOWLEDGMENTS

NP and PC are associates of Vaxine Pty Ltd, which owns the Advax™ adjuvant platform. Work described in this paper was funded by grants to Vaxine from Aus Industry through its Biotechnology Innovation Fund (BIF), START, Commercial Ready and Researcher-in-Business programs and by funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health under Contracts No. HHSN272200800039C and U01AI061142.

REFERENCES

1. Barclay T, Ginic-Markovic M, Cooper P, Petrovsky N. The chemistry and sources of fructose and their effect on functionality and health implications. *Journal of Excipients and Food Chemicals*. 2012; 3:67–82.
2. Leroux X. *La vie c'est bon comme tout*. Commercial Publication. 1996:5.
3. Robertfroid, M. *Inulin-Type Fructans: Functional Food Ingredients*. CRC Press; 2004.

4. Franck, A.; De Leenheer, L. Inulin. Polysaccharides and Polyamides in the Food Industry Properties, production and Patents. Wiley-VCH Verlag; 2005. p. 281-322.
5. Jackson KG, Taylor GR, Clohessy AM, Williams CM. The effect of the daily intake of inulin on fasting lipid, insulin and glucose concentrations in middle-aged men and women. *Br J Nutr.* 1999; 82:23–30. [PubMed: 10655953]
6. Daubioul C, Rousseau N, Demeure R, Gallez B, Taper H, Declerck B, et al. Dietary fructans, but not cellulose, decrease triglyceride accumulation in the liver of obese Zucker fa/fa rats. *J Nutr.* 2002; 132:967–73. [PubMed: 11983823]
7. Rault-Nania MH, Gueux E, Demougeot C, Demigne C, Rock E, Mazur A. Inulin attenuates atherosclerosis in apolipoprotein E-deficient mice. *Br J Nutr.* 2006; 96:840–4. [PubMed: 17092371]
8. Johnson IT. New approaches to the role of diet in the prevention of cancers of the alimentary tract. *Mutation research.* 2004; 551:9–28. [PubMed: 15225578]
9. Femia AP, Luceri C, Dolaro P, Giannini A, Biggeri A, Salvadori M, et al. Antitumorigenic activity of the prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* on azoxymethane-induced colon carcinogenesis in rats. *Carcinogenesis.* 2002; 23:1953–60. [PubMed: 12419846]
10. Petrovsky N. Immunomodulation with microbial vaccines to prevent type 1 diabetes mellitus. *Nature reviews Endocrinology.* 2010; 6:131–8.
11. Shannon JA, Smith HW. The Excretion of Inulin, Xylose and Urea by Normal and Phlorizinized Man. *J Clin Invest.* 1935; 14:393–401. [PubMed: 16694313]
12. British Pharmaceutical codex. 1979
13. Gotze O, Muller-Eberhard HJ. The C3-activator system: an alternate pathway of complement activation. *J Exp Med.* 1971; 134:90–108. [PubMed: 19867385]
14. Terman DS. Protein A and staphylococcal products in neoplastic disease. *Critical reviews in oncology/hematology.* 1985; 4:103–24. [PubMed: 3905035]
15. Cooper PD. Complement and cancer: activation of the alternative pathway as a theoretical base for immunotherapy. *Advances in immunity and cancer therapy.* 1985; 1:125–66. [PubMed: 3916662]
16. Phelps CF. The Physical Properties of Inulin Solutions. *Biochem J.* 1965; 95:41–7. [PubMed: 14333566]
17. Cooper PD, Steele EJ. Algamulin, a new vaccine adjuvant comprising gamma inulin particles containing alum: preparation and in vitro properties. *Vaccine.* 1991; 9:351–7. [PubMed: 1872020]
18. Cooper, PD. Activators and inhibitors of complement. SR, editor. Kluwer Academic Publishers, Dordrecht, Netherlands; Dordrecht: 1993. p. 69-106.
19. Cooper PD, Carter M. The anti-melanoma activity of inulin in mice. *Mol Immunol.* 1986; 23:903–8. [PubMed: 3540619]
20. Korbek M, Cooper PD. Potentiation of photodynamic therapy of cancer by complement: the effect of gamma-inulin. *British journal of cancer.* 2007; 96:67–72. [PubMed: 17146472]
21. Dempsey PW, Allison ME, Akkaraju S, Goodnow CC, Fearon DT. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science.* 1996; 271:348–50. [PubMed: 8553069]
22. Cooper PD, Steele EJ. The adjuvanticity of gamma inulin. *Immunol Cell Biol.* 1988; 66(Pt 5-6): 345–52. [PubMed: 3265692]
23. Silva DG, Cooper PD, Petrovsky N. Inulin-derived adjuvants efficiently promote both Th1 and Th2 immune responses. *Immunol Cell Biol.* 2004; 82:611–6. [PubMed: 15550119]
24. Kerekes K, Cooper PD, Prechl J, Jozsi M, Bajtay Z, Erdei A. Adjuvant effect of gamma-inulin is mediated by C3 fragments deposited on antigen-presenting cells. *J Leukoc Biol.* 2001; 69:69–74. [PubMed: 11200070]
25. Fanni D, Ambu R, Gerosa C, Nemolato S, Iacovidou N, Van Eyken P, et al. Aluminum exposure and toxicity in neonates: a practical guide to halt aluminum overload in the prenatal and perinatal periods. *World journal of pediatrics : WJP.* 2014; 10:101–7. [PubMed: 24801228]
26. Gherardi RK, Authier FJ. Aluminum inclusion macrophagic myofasciitis: a recently identified condition. *Immunol Allergy Clin North Am.* 2003; 23:699–712. [PubMed: 14753387]

27. Leventhal JS, Berger EM, Brauer JA, Cohen DE. Hypersensitivity reactions to vaccine constituents: a case series and review of the literature. *Dermatitis : contact, atopic, occupational, drug.* 2012; 23:102–9.
28. Tseng CT, Sbrana E, Iwata-Yoshikawa N, Newman PC, Garron T, Atmar RL, et al. Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus. *PLoS ONE.* 2012; 7:e35421. [PubMed: 22536382]
29. Cooper PD, Petrovsky N. Delta inulin: a novel, immunologically active, stable packing structure comprising beta-D-[2 -> 1] poly(fructo-furanosyl) alpha-D-glucose polymers. *Glycobiology.* 2011; 21:595–606. [PubMed: 21147758]
30. Cooper PD, Barclay TG, Ginic-Markovic M, Petrovsky N. The polysaccharide inulin is characterized by an extensive series of periodic isoforms with varying biological actions. *Glycobiology.* 2013; 23:1164–74. [PubMed: 23853206]
31. Andre I, Putaux JL, Chanzy H, Taravel FR, Timmermans JW, de Wit D. Single crystals of inulin. *International journal of biological macromolecules.* 1996; 18:195–204. [PubMed: 8729031]
32. Cooper PD, Barclay TG, Ginic-Markovic M, Gerson AR, Petrovsky N. Inulin isoforms differ by repeated additions of one crystal unit cell. *Carbohydrate polymers.* 2014; 103:392–7. [PubMed: 24528745]
33. Cooper PD, Rajapaksha KH, Barclay TG, Ginic-Markovic M, Gerson AR, Petrovsky N. Inulin crystal initiation via a glucose-fructose cross-link of adjacent polymer chains: atomic force microscopy and static molecular modelling. *Carbohydrate polymers.* 2015; 117:964–72. [PubMed: 25498723]
34. Pangburn MK, Ferreira VP, Cortes C. Discrimination between host and pathogens by the complement system. *Vaccine.* 2008; 26(Suppl 8):I15–21. [PubMed: 19388159]
35. Sahu A, Kozel TR, Pangburn MK. Specificity of the thioester-containing reactive site of human C3 and its significance to complement activation. *Biochem J.* 1994; 302(Pt 2):429–36. [PubMed: 8092994]
36. Murphy BF, Saunders JR, O'Bryan MK, Kirszbaum L, Walker ID, d'Apice AJ. SP-40,40 is an inhibitor of C5b-6-initiated haemolysis. *Int Immunol.* 1989; 1:551–4. [PubMed: 2489042]
37. Cooper PD, Barclay TG, Ginic-Markovic M, Petrovsky N. Gamma ray sterilization of delta inulin adjuvant particles (Advax) makes minor, partly reversible structural changes without affecting adjuvant activity. *Vaccine.* 2014; 32:552–7. [PubMed: 24342245]
38. Saade F, Honda-Okubo Y, Trec S, Petrovsky N. A novel hepatitis B vaccine containing Advax, a polysaccharide adjuvant derived from delta inulin, induces robust humoral and cellular immunity with minimal reactogenicity in preclinical testing. *Vaccine.* 2013; 31:1999–2007. [PubMed: 23306367]
39. Weeratna RD, Brazolot Millan CL, McCluskie MJ, Davis HL. CpG ODN can redirect the Th bias of established Th2 immune responses in adult and young mice. *FEMS Immunol Med Microbiol.* 2001; 32:65–71. [PubMed: 11750224]
40. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature.* 2008; 453:1122–6. [PubMed: 18496530]
41. Honda-Okubo Y, Saade F, Petrovsky N. Advax, a polysaccharide adjuvant derived from delta inulin, provides improved influenza vaccine protection through broad-based enhancement of adaptive immune responses. *Vaccine.* 2012; 30:5373–81. [PubMed: 22728225]
42. Murugappan S, Frijlink HW, Petrovsky N, Hinrichs WL. Enhanced pulmonary immunization with aerosolized inactivated influenza vaccine containing delta inulin adjuvant. *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences.* 2014; 66C:118–22. [PubMed: 25459531]
43. Honda-Okubo Y, Kolpe A, Li L, Petrovsky N. A single immunization with inactivated H1N1 influenza vaccine formulated with delta inulin adjuvant (Advax) overcomes pregnancy-associated immune suppression and enhances passive neonatal protection. *Vaccine.* 2014; 32:4651–9. [PubMed: 24958701]

44. Layton RC, Petrovsky N, Gigliotti AP, Pollock Z, Knight J, Donart N, et al. Delta inulin polysaccharide adjuvant enhances the ability of split-virion H5N1 vaccine to protect against lethal challenge in ferrets. *Vaccine*. 2011; 29:6242–51. [PubMed: 21736913]
45. Feinen B, Petrovsky N, Verma A, Merkel TJ. Advax-Adjuvanted Recombinant Protective Antigen Provides Protection against Inhalational Anthrax That Is Further Enhanced by Addition of Murabutide Adjuvant. *Clinical and vaccine immunology : CVI*. 2014; 21:580–6. [PubMed: 24554695]
46. Who. Japanese Encephalitis Vaccines: WHO position paper, February 2015 -Recommendations. *Vaccine*. 2015
47. Lobigs M, Pavy M, Hall RA, Lobigs P, Cooper P, Komiyama T, et al. An inactivated Vero cell-grown Japanese encephalitis vaccine formulated with Advax, a novel inulin-based adjuvant, induces protective neutralizing antibody against homologous and heterologous flaviviruses. *J Gen Virol*. 2010; 91:1407–17. [PubMed: 20130134]
48. Larena M, Prow NA, Hall RA, Petrovsky N, Lobigs M. JE-ADVAX Vaccine Protection against Japanese Encephalitis Virus Mediated by Memory B Cells in the Absence of CD8+ T Cells and Pre-Exposure Neutralizing Antibody. *Journal of virology*. 2013; 87:4395–402. [PubMed: 23388724]
49. Petrovsky N, Larena M, Siddharthan V, Prow NA, Hall RA, Lobigs M, et al. An inactivated cell culture Japanese encephalitis vaccine (JE-ADVAX) formulated with delta inulin adjuvant provides robust heterologous protection against West Nile encephalitis via cross-protective memory B cells and neutralizing antibody. *Journal of virology*. 2013; 87:10324–33. [PubMed: 23864620]
50. Bielefeldt-Ohmann H, Prow NA, Wang W, Tan CS, Coyle M, Douma A, et al. Safety and immunogenicity of a delta inulin-adjuvanted inactivated Japanese encephalitis virus vaccine in pregnant mares and foals. *Vet Res*. 2014; 45:130. [PubMed: 25516480]
51. Honda-Okubo Y, Barnard D, Ong CH, Peng BH, Tseng CT, Petrovsky N. Severe Acute Respiratory Syndrome-Associated Coronavirus Vaccines Formulated with Delta Inulin Adjuvants Provide Enhanced Protection while Ameliorating Lung Eosinophilic Immunopathology. *Journal of virology*. 2015; 89:2995–3007. [PubMed: 25520500]
52. Cristillo AD, Ferrari MG, Hudacik L, Lewis B, Galmin L, Bowen B, et al. Induction of mucosal and systemic antibody and T-cell responses following prime-boost immunization with novel adjuvanted human immunodeficiency virus-1-vaccine formulations. *J Gen Virol*. 2011; 92:128–40. [PubMed: 21169215]
53. Menon V, Whitney S, Francis J, Ajayi L, Petrovsky N, Montefiori D, et al. Characterization in rabbits & nonhuman primates of the neutralizing antibody response elicited by DNA & protein vaccination with SIVmac251 & SIVsmE660. *Retrovirology*. 2012; 9(Supp 2):43. [PubMed: 22632376]
54. Calderon-Gonzalez R, Marradi M, Garcia I, Petrovsky N, Alvarez-Dominguez C. Novel nanoparticle vaccines for listeriosis. *Human vaccines & immunotherapeutics*. 2015:0.
55. Calderon-Gonzalez R, Tobes R, Pareja E, Frande-Cabanes E, Petrovsky N, Alvarez-Dominguez C. Identification and characterisation of T-cell epitopes for incorporation into dendritic cell-delivered *Listeria* vaccines. *J Immunol Methods*. 2015; 424:111–9. [PubMed: 26031451]
56. Rodriguez-Del Rio E, Marradi M, Calderon-Gonzalez R, Frande-Cabanes E, Penades S, Petrovsky N, et al. A gold glyco-nanoparticle carrying a listeriolysin O peptide and formulated with Advax delta inulin adjuvant induces robust T-cell protection against listeria infection. *Vaccine*. 2015; 33:1465–73. [PubMed: 25659269]
57. Cosseddu GM, Polci A, Pinoni C, Capobianco Dondona A, Iapaolo F, Orsini G, et al. Evaluation of Humoral Response and Protective Efficacy of an Inactivated Vaccine Against Peste des Petits Ruminants Virus in Goats. *Transboundary and emerging diseases*. 2015
58. Eckersley AM, Petrovsky N, Kinne J, Wernery R, Wernery U. Improving the dromedary antibody response: The hunt for the ideal camel adjuvant. *J Camel Pract And Res*. 2011; 18:35–46.
59. Honda-Okubo Y, Ong CH, Petrovsky N. Advax delta inulin adjuvant overcomes immune immaturity in neonatal mice thereby allowing single-dose influenza vaccine protection. *Vaccine*. 2015

60. Cullen SP, Kearney CJ, Clancy DM, Martin SJ. Diverse Activators of the NLRP3 Inflammasome Promote IL-1beta Secretion by Triggering Necrosis. *Cell reports*. 2015; 11:1535–48. [PubMed: 26027935]
61. Marichal T, Ohata K, Bedoret D, Mesnil C, Sabatel C, Kobiyama K, et al. DNA released from dying host cells mediates aluminum adjuvant activity. *Nat Med*. 2011; 17:996–1002. [PubMed: 21765404]
62. Schuster JM, Nelson PS. Toll receptors: an expanding role in our understanding of human disease. *J Leukoc Biol*. 2000; 67:767–73. [PubMed: 10857847]
63. Gordon D, Kelley P, Heinzel S, Cooper P, Petrovsky N. Immunogenicity and safety of Advax, a novel polysaccharide adjuvant based on delta inulin, when formulated with hepatitis B surface antigen: A randomized controlled Phase 1 study. *Vaccine*. Sep 27. pii. 2014; S0264-410X(14): 01293–6. doi: 10.1016/j.vaccine.2014.09.034.
64. Gordon DL, Sajkov D, Woodman RJ, Honda-Okubo Y, Cox MM, Heinzel S, et al. Randomized clinical trial of immunogenicity and safety of a recombinant H1N1/2009 pandemic influenza vaccine containing Advax polysaccharide adjuvant. *Vaccine*. 2012; 30:5407–16. [PubMed: 22717330]
65. Heddle R, Russo P, Petrovsky N, Hanna R, Smith A. Immunotherapy – 2076. A controlled study of delta inulin-adjuvanted honey bee venom immunotherapy. *The World Allergy Organization journal*. 2013; 6:P158–P.

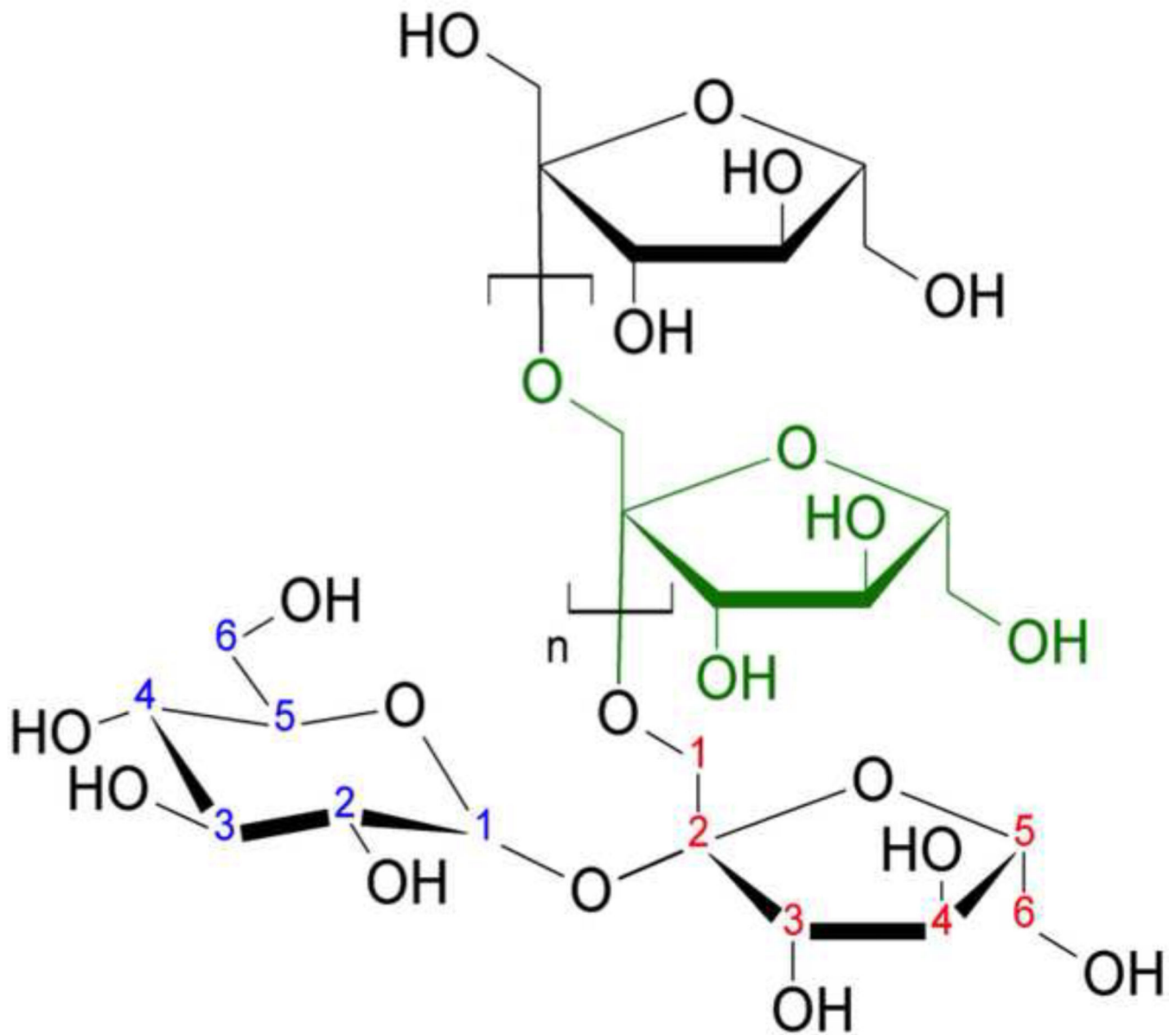


Figure 1. Structure of a single inulin polymer

This shows inulin comprises a variable-length chain of fructose rings terminated with a single glucose ring. In general, inulin polymers isolated from plant sources range in length from 3 – 100 fructose units.

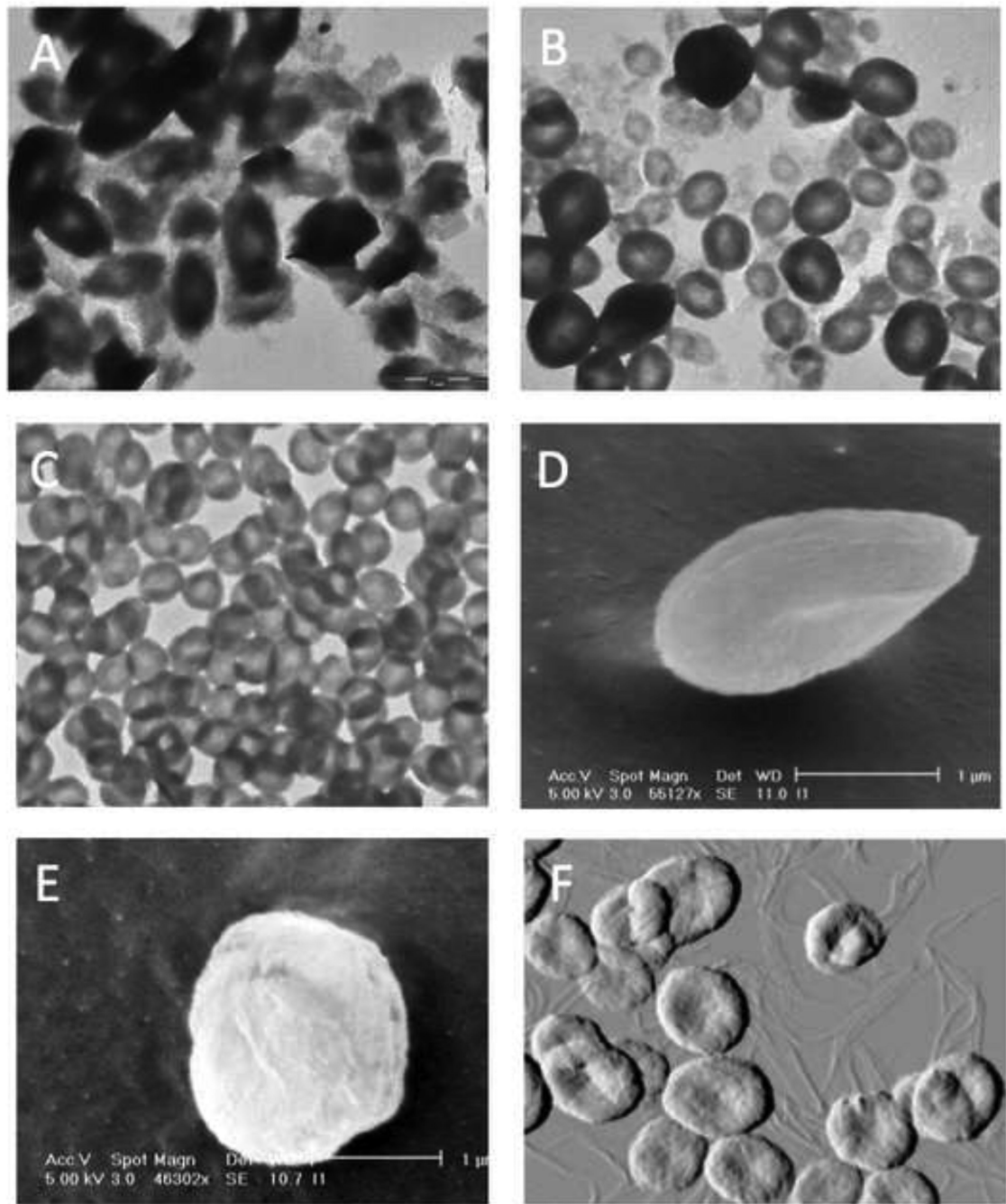


Figure 2. Microscopic structures of crystalline inulin particles

Transmission electron microscopy (TEM) reveals gamma inulin particles to have highly variable shape, size and structure (A) potentially explaining their large batch-to-batch variation in adjuvant activity. Initial delta inulin particles had better defined structure on TEM but still exhibited considerable size variation (B). Optimization and control of crystallization parameters allowed reproducible production of delta inulin particles of consistent size and shape (C) that subsequently formed the basis of Advax™ adjuvant. Freeze-fracture scanning EM of Advax™ particles suspended in water reveals their discoid

shape and formation from lamellar sheets (D&E). Atomic force microscopy of Advax™ particles during their formation confirms their discoid character and also fine inulin filament precursors from which the final particles are assembled (F).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript