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CHAPTER 10

Advax Adjuvant: A Potent and Safe Immunopotentiator Composed of Delta Inulin

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DELTA INULIN BACKGROUND

Inulin is a plant-based polysaccharide composed of β(2→1) polyfructofuranosyl α-D-glucose polymer chains in which a linear chain of fructose rings is terminated by a glucose ring (Fig. 10.1A). Plants of the Compositae family (dahlias, chicory, artichoke, onions, garlic) produce inulin as a storage carbohydrate (reviewed in[1]). Inulin's medicinal uses date back to ancient times; as early as AD 100 Pedanios Dioscoride, a Roman army physician, identified the beneficial effects of chicory root extract (~40% inulin by weight) for the treatment of stomach, liver, and kidney complaints.[2] In 1804, inulin was purified from a boiling water extract of *Inula helenium*[3] leading to its current name.[4] The beneficial effects of inulin were rediscovered in 1874 when it was shown diabetic patients put on a daily diet of 100 g of inulin lost their glycosuria.[3] Dietary inulin has been shown to reduce fasting insulin, triacylglycerol,[5] hepatic lipogenesis,[6] and atherosclerotic lesions[7];

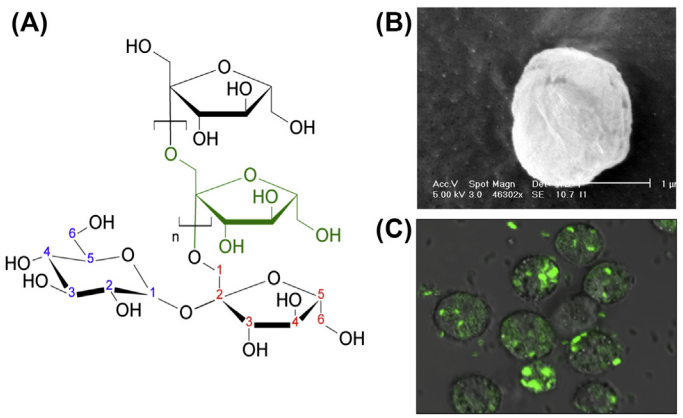

Figure 10.1 Structure of delta inulin. Schematic of a single inulin polymer chain composed of multiple fructose units with a terminal glucose (A). A single particle of delta inulin imaged by freeze-fracture scanning electron microscopy (B). Fluorescent-labelled particles of delta inulin endocytosed by human monocytes after overnight co-culture (C).

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as well as enhance expression of histone deacetylases; and induce apoptosis of colon cancer cells.[8] It also has antiinflammatory effects and has been shown to suppress autoimmune disease.[9] The inability of mammals to metabolize inulin led in the 1920s to its use for the measurement of glomerular filtration, confirming its exceptional safety including in pregnant women and newborn babies.[10] Injection of inulin has no adverse effects other than a mild osmotic diuresis.[11] Some patients injected with inulin developed transient hypotensive symptoms, found to reflect complement activation, a finding that led to the discovery of the alternative complement pathway (ACP).[12] The ability of crystalline (gamma) inulin to activate the ACP was initially exploited as an anticancer treatment.[13–17] However, the connection between complement activation and adaptive immunity promted the testing inulin particles for their ability to enhance vaccine immunogenicity. Inulin particles turned out to have surprisingly potent immune modulatory properties, an attribute not shared by soluble inulin. This led to development of delta inulin (Advax) as a highly stable well-defined microcrystalline inulin particle (Fig. 10.1B) with potent vaccine adjuvant action, as described below.

MANUFACTURE OF DELTA INULIN

Inulin has a hydrophobic polyoxyethylene-like backbone that is critical to its structure in solution and when crystallized.[18–21] Inulin particles obtained by precipitation from cold water are referred to as alpha inulin, the particles obtained by precipitation from ethanol are called beta inulin, and particles obtained by precipitation from water at progressively higher temperatures are called gamma and delta inulin.[19] Delta inulin is distinguished from earlier inulin isoforms by being insoluble at temperatures below 40°C, an important distinction as it means that delta inulin particles are insoluble at body temperatures.[21] Each delta inulin particle is made up of individual inulin polymers each formed into an antiparallel double helix, with these helixes then assembling together into lamellar sheets through lateral hydrogen bonding.[18–21] Imaging studies of delta inulin particles show spherulite-like discoid particles.[22] Consistent with its stability, delta inulin is resistant to damage from sterilizing doses of gamma irradiation to which gamma inulin is highly susceptible.[23] Delta inulin particles manufactured under current Good Manufacturing Practices (cGMP) have a highly consistent size, shape, and immune properties, in contrast to the high variability of naturally occurring inulin particles. The terms Advax and delta inulin are not strictly synonymous with Advax being composed of delta inulin particles of a specific particle size and morphology made under cGMP and optimized for maximal adjuvant activity.

ADVAX-ADJUVANTED HEPATITIS B VACCINE

Mice injected intramuscularly or subcutaneously with hepatitis B surface antigen (HBsAg) plus delta inulin showed ~ fourfold antigen dose sparing versus an alum-adjuvanted

HBsAg vaccine.[19] When compared with the Th2 bias of alum adjuvant, delta inulin delivered a more balanced Th1/Th2 response with T-cell recall assays revealing increased production of a broad range of cytokines including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-5, IL-6, IL-10, IL-13, IL-17, and granulocyte-macrophage colony-stimulating factor (GM-CSF).[24] Interestingly, IL-1α production was not increased by Advax, indicating that, unlike alum,[25] delta inulin does not activate the inflammasome.[24] Delta inulin's immune potentiator effect was not dependent on antigen adsorption as Advax could be injected one day prior to the HBsAg and still potentiated antibody production, unlike an alum comparator.[24] Interestingly, when injected on the opposite side of the body to the antigen, Advax reduced the antibody response, implying a potential immune decoy effect.[24] In guinea pigs Advax similarly induced higher anti-HBsAg IgG titers and accelerated seroprotection by several days when compared with an alum-adjuvanted vaccine.[24] A first-in-man phase 1 clinical trial assessed the safety and tolerability of Advax-adjuvanted HBsAg vaccine (Table 10.1). Adult subjects received three intramuscular doses of a commercial HBsAg vaccine antigen supplied by Butantan Institute, alone or formulated with Advax adjuvant.[26] Advax significantly increased antibody levels and anti-HBsAg CD4+ T-cell responses. By comparison, the majority of subjects immunized with antigen alone did not achieve seroprotection or measurable T-cell responses after three immunizations. Advax was well tolerated with no significant differences in injection site pain scores or adverse events when compared to injection of HBsAg alone.

ADVAX-ADJUVANTED INFLUENZA VACCINES

Most inactivated or recombinant influenza vaccines are currently administered without adjuvant and only provide partial protection against influenza infection. Addition of Advax adjuvant to trivalent inactivated influenza vaccine (Fluvax, CSL Australia) or a monovalent inactivated H1N1 influenza virus antigen (A/Puerto Rico/8/1934) enhanced neutralizing antibody titers and provided up to 100-fold antigen sparing in mice.[27] This correlated with a higher frequency of IgM- and IgG-secreting memory B cells in the bone marrow and spleen. Advax increased influenza-specific CD4+ and CD8+ T-cell proliferative responses and IFN-γ, IL-2, IL-5, IL-6, and GM-CSF production, and this translated into enhanced protection against a lethal influenza challenge. The effect of Advax on immune memory was long lived with elevated antibody titers, T-cell responses, and protection still evident 1 year postimmunization.[27] Injection of mice with Advax alone in absence of antigen provided no protection against influenza infection, indicating Advax, unlike Toll-like receptor (TLR) agonists, does not activate nonspecific innate immune pathways. Advax was also tested for its ability to help overcome the negative effects of pregnancy on influenza vaccine responses. Pregnant dams immunized intramuscularly with a single dose of inactivated influenza antigen with Advax had higher

Table 10.1 Listing of Human Vaccine Trials Incorporating Advax Adjuvant

Registered Trial Number	Indication	Comparator Groups	Start Year	Trial Title	Trial Sites	Subjects	Trial Status
ACTRN 12607000598482	Hepatitis B prophylaxis	Antigen alone Advax	2006	A randomized, controlled vaccine intervention study in healthy adults evaluating the safety and immunogenicity of hepatitis B vaccine containing Advax adjuvant (HBV001)	Australia	Males and females 18–40 years	Completed
ACTRN 12607000599471	Seasonal influenza prophylaxis	Antigen alone Advax	2007	A randomized, controlled phase 1/2 study in healthy adults of a seasonal influenza vaccine containing an inulin-based adjuvant to evaluate safety and immunogenicity (FLU001)	Australia	Males and females 18–70 years	Completed
ACTRN 12608000364370	Seasonal influenza prophylaxis	Antigen alone Advax	2008	Randomized, controlled phase 1/2 study to evaluate the safety and effectiveness of the enhanced potency of adjuvanted seasonal influenza vaccine in patients with chronic disease and the elderly (FLU002)	Australia	Males and females 20–85 years	Completed
ACTRN 12608000350325	Seasonal influenza prophylaxis	Antigen alone Advax	2008	A randomized, controlled phase 1/2 study in healthy adults to evaluate the safety and immunogenicity of an inulin-adjuvanted antigen-sparing seasonal trivalent inactivated influenza vaccine (FLU004)	Australia	Males and females 18–70 years	Completed
ACTRN 12608000379314	Bee sting anaphylaxis therapy	Antigen alone Advax	2008	A randomized, controlled phase 1/2 study of adults with bee venom allergy to evaluate the safety and efficacy of an Advax-adjuvanted bee venom desensitization regimen (BEE001)	Australia	Males and females 18–65 years	Completed

ACTRN 12609000674235	Pandemic influenza prophylaxis	Antigen alone Advax	2009	A randomized, controlled, study in healthy adults to evaluate the safety and immunogenicity of an adjuvanted recombinant 2009 H1N1 pandemic swine influenza vaccine (FLU005)	Australia	Males and females 18–90 years	Completed
ACTRN 12612000709842	Seasonal influenza prophylaxis	Antigen alone Advax	2012	A randomized controlled trial to evaluate the immune response to an adjuvanted 2012 seasonal trivalent inactivated influenza vaccine in adults delivered via needle and syringe or jet injector device (FLU006)	Australia	Males and females 18 years +	Completed
ClinicalTrials.gov NCT01677676	Universal influenza vaccine	Antigen alone Advax	2012	Safety, tolerability, and immunogenicity of two different formulations of an influenza A vaccine (FP-01.1_CS_02)	Australia	Male and female 18–55 years	Completed
ClinicalTrials.gov NCT01701752	Universal influenza vaccine	Antigen alone Advax	2013	Influenza A vaccine (FP-01.1) formulated with and without adjuvant, in the presence or absence of a single administration of a trivalent inactivated influenza virus vaccine in older adults (FP-01.1_CS_03)	Belgium	Male and female 55–75 years	Completed
ClinicalTrials.gov NCT01951677	Hepatitis B prophylaxis	Antigen alone Advax, Advax-SM Alum	2014	Single-center, randomized, controlled, blinded phase 1/2 study to compare the safety and effectiveness of hepatitis B vaccine formulations in individuals with renal impairment, diabetes mellitus, or age greater than 40 years (HBV002)	Australia	Male and female 18 + years	Recruiting
ClinicalTrials.gov NCT02335164	Pandemic influenza prophylaxis	Antigen alone Advax Advax-SM	2015	A randomized, controlled, phase 1 study to evaluate the immunogenicity and safety of a pandemic avian H5 influenza vaccine in adult subjects (FLU003)	Australia	Male and female 18 + years	Recruiting

serum antiinfluenza IgG titers and this translated into higher influenza-specific IgG titers in their breast milk and higher IgG titers in the serum of their suckling pups, which were completely protected when challenged with a lethal influenza challenge at 4 weeks of age.[28] Advax was well tolerated by the pregnant dams and no adverse effects were observed on mothers or their pups. Other adjuvants, namely, alum[29] and MF59 squalene emulsion[30] failed to protect neonates, even when given as a two-dose vaccine regimen at 7 and 21 days of age. The ability of Advax to enhance influenza vaccine protection was therefore tested in 7-day-old pups. Immunization of 7-day-old pups with a single dose of influenza antigen with Advax increased serum IgM and IgG1 levels, memory B and T cell frequency, and protected against influenza challenge at 28 days of age.[31] Remarkably, given that neonatal T cells have been described to have a fixed defect in the ability to make IFN-γ irrespective of stimulus,[32] Advax restored the ability of neonatal T cells to make IFN-γ recall responses. The benefits of Advax are not just restricted to seasonal influenza vaccines as in a ferret H5N1 challenge model, Advax enhanced protection against high-pathogenicity H5N1 avian influenza.[33] Reduced clinical disease, absence of brain invasion, faster virus clearance, and complete survival were seen in the Advax-adjuvanted groups.

Advax is not only effective when given via parenteral routes. In an alternative delivery approach, Advax was well tolerated and boosted the immunogenicity of an inactivated influenza antigen delivered directly into the lung via intratracheal spray.[34]

Advax adjuvant has been tested in multiple human influenza vaccine trials (Table 10.1). For example, Advax was shown in adult human subjects to significantly enhance the immunogenicity of Panblok (Protein Sciences Corporation, Meriden), a pandemic influenza vaccine based on recombinant hemagglutinin.[35] Advax increased seroprotection rates twofold and was well tolerated with no safety issues. Interestingly, the inclusion of Advax was associated with a significant ~75% reduction in subjects experiencing headaches postimmunization as recorded in subject diaries, hypothesized to be due to Advax inhibiting IL-1 production.[35]

OTHER ADVAX-ADJUVANTED VACCINES

Advax has been shown to be effective in increasing the immunogenicity of a broad range of viral, bacterial, parasitic, and toxin vaccines. For example, Advax enhanced the efficacy of a recombinant protective antigen vaccine against pulmonary anthrax, increasing neutralizing antibody levels and protecting against an aerosol challenge, even after just a single immunization.[36] Advax had a synergistic adjuvant effect with murabutide, a NOD2 agonist. As shown by in vivo imaging of cathepsin cleavage, the combined Advax and murabutide adjuvant formulation induced significantly less injection site inflammation than an alum adjuvant comparator.

Similarly, inclusion of Advax in an inactivated Japanese encephalitis (JEV) vaccine enhanced neutralizing antibody levels in immunized mice and horses.[37] Animals immunized with Advax-adjuvanted JEV vaccine also exhibited cross-neutralizing antibody responses and protection against Murray Valley encephalitis virus and West Nile virus (WNV) infection, an effect shown to be mediated by cross-protective memory B cells.[38] After just a single vaccine dose containing Advax, WNV cross-protection was still evident at 1 year postimmunization.[39] This JEV vaccine was shown to be safe when administered to pregnant mares and newborn foals, in which it similarly induced cross-neutralizing antibody responses.[40]

In another example, Advax increased neutralizing antibody levels, CD4 and CD8 T-cell proliferative responses, and vaccine protection in a murine model of severe acute respiratory syndrome (SARS) coronavirus infection.[41] An alum-formulated SARS vaccine resulted in increased eosinophilic lung immunopathology after SARS virus challenge, whereas lung immunopathology was reduced in mice that received Advax-adjuvanted vaccine. Advax induced a high frequency of IFNγ-producing SARS-specific T cells and inhibition of SARS-associated lung immunopathology was further enhanced by coformulation of Advax with CpG oligonucleotide.

Advax alone or in combination with CpG oligonucleotide similarly enhanced the immunogenicity of a human immunodeficiency virus envelope vaccine in mice, inducing persistent mucosal gp120-specific IgA, systemic IgG, and memory T- and B-cell responses.[42] It similarly enhanced the production of simian immunodeficiency virus neutralizing antibodies in rabbits and nonhuman primates when combined with an envelope protein boost following a DNA vaccine prime.[43]

Advax enhanced protection by a *Listeria* vaccine based on T-cell peptide epitopes conjugated to gold nanoparticles.[44,45] This correlated with an increased frequency of splenic CD4+ and CD8+ T cells, NK cells, and CD8α+ dendritic cells plus increased Th1 cytokine production (IL-12, IFN-γ, TNF-α, and MCP1 [macrophage chemotactic protein (MCP)-1]) and was associated with increased T-cell epitope spreading following live *Listeria* challenge.[46]

Advax also increased neutralizing antibody responses and protection in goats immunized against Peste de petit ruminants.[47] Consistent with broad species activity, it was also effective in camels when used with vaccines against African horse sickness and meliodosis.[48]

MECHANISM OF ACTION

Most adjuvants work via induction of danger signals. Alum induces inflammasome activation and cell death[49] with DNA released from lysed cells resulting in TLR activation.[50] Monophosphoryl lipid A activates TLR4, flagellin activates TLR5, and CpG oligonucleotides activate TLR9. Hence, these adjuvants all induce activation of nuclear factor-κB

and an inflammatory response.[51] Advax does not induce inflammatory cytokine production, thereby distinguishing it from other adjuvants. Although its mechanism of action remains the subject of intense ongoing study, it is hypothesized to work by modulating antigen-presenting cell function in a noninflammatory manner, thereby enhancing the costimulation and activation of antigen-specific helper T cells that in turn license the expansion of memory B cells and CD8 T cells. In human subjects receiving seasonal influenza vaccine Advax enhanced the day 7 plasmablast response, and enhanced influenza-specific B-cell affinity maturation, as reflected by a higher rate of nonsilent mutations in the B-cell receptor CDR3 region. This was associated with higher levels of activation-induced cytidine deaminase in the day 7 plasmablasts from subjects receiving Advax, thereby explaining the higher-avidity IgG levels.[52]

PRECLINICAL SAFETY AND TOXICOLOGY

A range of single and repeated dose GLP safety studies of Advax have been undertaken in guinea pigs, rats, and rabbits in combination with a range of inactivated and recombinant vaccine antigens. These studies have all confirmed the lack of significant local and systemic reactogenicity of Advax-adjuvanted vaccines. Histology of the injection site in these studies revealed a collection of macrophages and neutrophils, as typically seen with vaccine injections, but without any muscle necrosis or overlying skin swelling or heat to suggest overt inflammation. Similarly, even when given at high doses Advax did not induce pyrexia, and there were minimal changes to acute-phase reactants, serum inflammatory markers, and hematological or biochemical parameters in response to Advax administration. Although typical pharmacokinetic studies are not required for licensing of vaccines, long-term tissue residence of adjuvants such as alum and oil emulsions has been suggested to contribute to potential toxicity. Studies undertaken into the biodistribution and excretion of parenterally administered Advax have shown that it is rapidly excreted through the urine. Hence, the majority of an intramuscular injection of Advax in mice was shown to be cleared through the urine within 7 days.

CONCLUSIONS

Advax has been shown to enhance the immunogenicity of a broad range of antigens including whole inactivated viruses, recombinant proteins, synthetic peptides, toxins, and venoms. It is effective in a broad range of animal species and is safe and well tolerated when administered during pregnancy and early neonatal life. It acts synergistically with traditional innate immune activators such as murabutide or CpG oligonucleotides. Positive results in preclinical studies have translated into successful human clinical trials, which have confirmed its exceptional safety, tolerability, and efficacy.

Given its many benefits, which include enhancement of humoral and cellular immunity, ability to overcome neonatal immune immaturity and pregnancy-associated immunosuppression, lack of reactogenicity, rapid excretion, and strong safety, Advax could be ideal for use in pediatric vaccines and vaccines for pregnant women. When formulated with SARS coronavirus vaccine, Advax reduced the risk of lung eosinophilic immunopathology, suggesting that it may be beneficial for use in respiratory virus vaccines, e.g., against SARS, Middle East respiratory syndrome and respiratory syncytial virus, where the risk of vaccine-enhanced lung immunopathology is a major concern. Advax's ability to enhance both humoral and cellular immunity makes it particularly suited for use in antiviral vaccines, given the important role of T cells in clearing viral infections. Its ability to induce high numbers of antigen-specific IFN-γ-producing T cells also makes Advax well suited for use in vaccines against intracellular pathogens, such as *Listeria* or *Mycobacterium tuberculosis*. Other areas of promise are use in allergy vaccines to accelerate desensitization, and in vaccines against chronic viral infections where T-cells are needed for control of viral replication.

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