Chapter 7
Sugar-Based Immune Adjuvants for Use in Recombinant, Viral Vector, DNA and Other Styles of Vaccines

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Abstract Highly attenuated viral vectors, purified protein antigens and DNA vaccines all suffer from problems of low immunogenicity providing a major challenge to find the best way to address this problem. A convenient solution is to identify a suitable adjuvant to add to the vaccine formulation to enhance its immunogenicity. Adjuvants come in many shapes and flavours, with no single unifying theme to explain why such a diversity of compounds should share the ability to enhance vaccine action. Hence adjuvant selection remains an empiric exercise of trial and error, largely based on comparisons of adjuvant potency in animal models plus assessment of safety and tolerability. In addition, there are unique challenges with successfully adjuvanting nonprotein-based vaccine technologies such as viral vectors or DNA vaccines for which existing adjuvant technologies such as aluminium salts or oil emulsions are likely to be unsuitable or incompatible. Amongst the many different groups of potential adjuvant compounds, carbohydrate-based adjuvants have received relatively little attention despite having favourable properties including compatibility for formulation with live vector vaccines, safety, tolerability and ease of manufacture and formulation. These properties may make them ideal for use across all vaccine platforms including live viral vectors and DNA vaccines. This chapter will review the various carbohydrate-based adjuvants and the science that underpins their activity and will highlight the potential for sugar-based adjuvants to replace more traditional adjuvant such as aluminium salts and oil emulsions across a wide variety of human and veterinary vaccine applications using traditional antigens, viral vectors or DNA to protect against infectious disease and for treatment of cancer and allergy.

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7.1 Introduction

The goal of vaccination is to generate a protective immune response of sufficient strength and duration to prevent or attenuate the virulence of pathogenic organisms. This is achieved by immunisation using either inactivated or recombinant proteins or sugars, live attenuated vectors or DNA plasmid vaccines targeting key antigens expressed by the pathogen or, in the case of cancer vaccines, the particular tissue being targeted. The aim is to induce a protective immune response that may comprise a neutralising antibody response or cytotoxic T-cell response against the relevant target. In the early days of vaccinology, inactivated whole cell antigens being impure and containing immunologically active contaminants generated strong immunity without the need for an added adjuvant. Thus, older-style attenuated virus vaccines such as the yellow fever vaccine were highly immunogenic on their own. On the negative side, early vaccines suffered from major reactogenicity and safety issues. Attempts in recent years to improve the quality and purity of vaccine antigens and vectors to reduce their reactogenicity and improve their safety have had the unfortunate consequence that vaccine immunogenicity has been correspondingly reduced. This has necessitated a search for ways to improve vaccine immunogenicity. The most commonly adopted approach has been to find an adjuvant that when added to the vaccine improves its immunogenicity and thereby its ability to protect against the relevant infectious disease or cancer.

An adjuvant is defined as any compound that enhances the immune response against a vaccine antigen; the word adjuvant comes from *adjuvare*, meaning to help or to enhance. Adjuvants can be used for multiple purposes: to enhance immunogenicity, provide antigen-dose sparing, accelerate the immune response, reduce the need for booster immunisations, increase the duration of protection, improve efficacy in poor responder populations or in the case of cancer vaccines to help break self-tolerance (Petrovsky and Aguilar 2004). To this day, alum-based adjuvants hold a relative monopoly over approved human vaccines although oil emulsion adjuvants are used to a limited extent in influenza vaccines. The reason for alum's dominance effects its long record of safe use, with newer adjuvants facing major regulatory hurdles to gain approval. Regulatory requirements require any new adjuvant to prove its benefits outweigh any safety or tolerability issues, making it difficult for new adjuvants to challenge alum's supremacy (Petrovsky 2013). However, approved adjuvants have largely proved unsuitable for use in viral vector vaccines, necessitating development of novel adjuvant approaches for enhancement of such vaccines. An additional factor driving the need for new adjuvants is that viral vector vaccines are not just administered through traditional parenteral injection approaches, but also through alternative routes such as intranasal or transdermal delivery for which parenteral adjuvants such as alum are not suited. Furthermore, safety concerns have been raised even with regard to alum (Passeri et al. 2011) and squalene emulsion adjuvants (Miller et al. 2013), making it critical that any new adjuvant have strong human safety and tolerability data

(Petrovsky 2008). Carbohydrate compounds such as polysaccharides are generally regarded as safe with minimal risk of toxic metabolites or long-term tissue deposits. From a safety aspect, this makes them ideal candidates amongst which to search for adjuvant-active compounds. Interestingly, many sugars play major signalling roles within the immune system, and hence it is not surprising that many carbohydrate compounds, when tested, have been found to have adjuvant activity (summarised in Table 7.1).

7.2 Fructan Adjuvants

One of the first polysaccharides recognised to have immunological effects was β-D-[2-1] poly(fructo-furanosyl)-D-glucose, more commonly known as inulin, a natural plant-derived storage carbohydrate of plants of the Compositae family. Inulin is a polymer comprising linear chains of fructosyl groups linked by β(2-1) glycosidic bonds terminated at the reducing end by an α-D-(1-2)-glucopyranoside ring group. Inulin's immune activity was first identified when inulin solutions being administered to human subjects to measure kidney function were noted to activate complement through a nonclassical antibody-independent pathway, thereby leading to the discovery of the alternative complement pathway. Complement activation was found to be due to minute inulin crystals contaminating inulin solutions (Cooper and Carter 1986). These insoluble inulin crystals with immunological activity need to be distinguished from the more soluble alpha- and beta-inulin forms that do not have immunological activity (Cooper et al. 2013). The most advanced inulin crystalline form, delta inulin, developed under the trade name, Advax™, was shown to have potent adjuvant activity (Cooper and Petrovsky 2011). Advax adjuvant has been demonstrated to enhance the immunogenicity of a wide variety of protein antigens drawn from viral, bacterial and protozoan pathogens, toxins, cancer antigens and allergens when administer to a broad range of animal species including mice, rats, guinea pigs, rabbits, chickens, dogs, sheep, monkeys, horses and camels. Advax has been shown to enhance vaccine immunogenicity in murine models of H1N1 influenza (Honda-Okubo et al. 2012), Japanese encephalitis (Larena et al. 2013), West Nile virus (Petrovsky et al. 2013), hepatitis B virus (Saade et al. 2013) and human immunodeficiency virus (Cristillo et al. 2011), a ferret model of avian (H5N1) influenza (Layton et al. 2011), a horse model of Japanese encephalitis (Lobigs et al. 2010) and camel models of African horse sickness and glanders (Eckersley et al. 2011). In the Japanese encephalitis and West Nile virus models, the enhanced protection obtained with Advax adjuvant was shown to be mediated by its ability to induce a protective memory B-cell population, whereas in other models such as influenza protection was shown to be mediated by both memory B-cell and T-cell populations. More recently, Advax adjuvant was effective in human trials where it significantly boosted seroconversion and seroprotection to a recombinant pandemic influenza vaccine (Gordon et al. 2012). A notable feature of Advax adjuvant is its lack of reactogenicity and

Table 7.1 Examples of carbohydrate-based adjuvants

Adjuvant name	Chemical structure	Modified forms	Source	Receptor(s)	Immune actions/mechanism of action
Delta inulin	β-D-[2-1]Poly (fructo-furanosyl) α-D-glucose	Epsilon, omega	Compositae plants	Not known	Noninflammatory, TLR-independent, activates complement, chemokine secretion, costimulation
Dextran	α-1,6-Glucan with α-1,3-branches	Dextran sulphate, DEAE-dextran, acetylated dextran	Lactobacilli	Glucan receptor	Proinflammatory, activates inflammasome, complement, NFkB, induces IL1β, IL-6, TNF-α
Lentinan	β-1,3-Glucohexaose with β-1,6-branches	Sulphated lentinan	Shiitake mushroom	Glucan receptor	Proinflammatory, activates inflammasome, complement, NFkB, induces IL1β, IL-6, TNF-α
Zymosan	β-1,3-Glucan	Glucan particles	*Saccharomyces cerevisiae*	GR, TLR2, Dectin-1, ASC	Proinflammatory, activates inflammasome, complement, NFkB, induces IL1β, IL-6, TNF-α
Beta-glucan	β-1,3-Glucan	Glucan particles	*Saccharomyces cerevisiae*	GR, TLR2, Dectin-1, ASC	Proinflammatory, activates inflammasome, complement, NFkB, induces IL1β, IL-6, TNF-α
Mannan	1,4-Polymaltose	Oxidation, reduction, acylation, mannosylated niosomes	*Aloe barbadensis*	MBL, mannose receptor	Proinflammatory, activates NFkB, induces IL1β, IL-6, TNF-α
Chitin	*N*-Acetyl-D-glucosamine	Acetylation (chitosan)	Crustaceans	Dectin-1, MMR, TLR-2	Proinflammatory, phosphorylates MAPK, induces TNF-α, COX-2 , prostaglandin E2, DC maturation
Muramyldipeptide	*N*-Acetyl muramyl-L-alanine-D-isoglutamine	D-Murapalmitine, GMDP, murabutide	Mycobacteria, *Lactobacillus bulgaricus*	NOD2	Proinflammatory, activates NFkB, induces IL1β, IL-6, TNF-α
Cord factor	Trehalose-6-6-dimycolate	Multiple	*M. tuberculosis*	Mincle	Proinflammatory, activates monocyte Syk-Card9 signalling, activates NFkB IL1β, IL-6, TNF-α

LPS	Lipopolysaccharide	Monophosphoryl lipid A (MPL)	Gram negative bacteria	TLR4	Proinflammatory, activates NFkB, induces IL1β, IL-6, TNF-α
QS21	Triterpenoid glycosides	GPI-0100	Bark of *Quillaja saponaria*	Inflammasome	Proinflammatory, activates inflammasome and NFkB

safety as demonstrated in many animal and human studies. This supports the hypothesis that polysaccharides on the whole are extremely well tolerated. Advax adjuvant induces robust CD4 and CD8 T-cell immunity against co-administered antigens (Honda-Okubo et al. 2012), raising potential for its use in cancer vaccines and vaccines for problematic infectious diseases such as HIV and tuberculosis where T-cell immunity is critical for protection. An interesting feature of Advax adjuvant is its ability to enhance adaptive immune responses even when injected separately in time to the antigen. Thus, the adjuvant action of Advax was maintained even when it was injected 24 h prior to injection of hepatitis B surface antigen, a feature not shared by alum adjuvant (Saade et al. 2013). Unlike alum, Advax does not work by binding antigen and forming a tissue depot, meaning formulation is a simple matter of mixing the antigen with Advax. The combined formulation can then be immediately injected if desired. Formulation is a particularly important aspect of preparation of viral vector vaccines as it is critical that virus viability not be adversely affected during storage with adjuvant. Recent studies confirmed that co-formulation with Advax did not adversely affect the viability of live vector vaccines including a live modified vaccinia Ankara (MVA) smallpox vaccine and a live respiratory syncytial virus (RSV) vaccine. Enhancement of vaccine immunogenicity was observed in both studies, confirming that delta inulin adjuvant can indeed enhance the immunogenicity of live viral vector vaccines. A critical question is how delta inulin is able to mediate these beneficial effects while not causing the reactogenicity and toxicity observed with other adjuvants. The difference may be that delta inulin does not work through activation of innate immune receptors such as the TLRs, Dectin-1 or the inflammasome and thereby does not induce proinflammatory and pyrogenic cytokines such as interleukin (IL)-1 that mediate side effects of others adjuvants. Delta inulin modulates antigen-presenting cell function, inducing phenotypic changes associated with enhanced antigen presentation to T and B cells, while bypassing innate immune activation thereby explaining the lack of reactogenicity.

7.3 Glucan Adjuvants

Glucans are plant- or microorganism-derived polysaccharides made up of repeating D-glucose units joined by glycosidic bonds in various alternative conformations. Alpha-glucans include dextran (α-1,6-glucan), glycogen (α-1,4- and α-1,6-glucan), pullulan (α-1,4- and α-1,6-glucan) and starch (α-1,4- and α-1,6-glucan). Beta-glucans include cellulose (β-1,4-glucan), curdlan (β-1,3-glucan), laminarin (β-1,3- and β-1,6-glucan), chrysolaminarin (β-1,3-glucan), lentinan (purified β-1,6:β-1,3-glucan from *Lentinus edodes*), lichenin (β-1,3- and β-1,4-glucan), pleuran (β-1,3- and β-1,6-glucan isolated from *Pleurotus ostreatus*) and zymosan (β-1,3-glucan from *Saccharomyces*). Each type and source of glucan can be of widely varying quality and purity and may contain mixtures of polymer chain

structures with differing amounts of branching and variation in chain length. Because these polymer variables are influenced by the source of the glucan, glucans are named according to their plant or microbial source. Hence while zymosan is predominantly β-1,3-glucan extracted from yeast cell walls, it also contains variable amounts of other sugars and yeast proteins that also have immunological activity. Hence, it is not always clear which component of complex glucan formulations is responsible for their adjuvant activity. As detailed in Table 7.1, glucans and other carbohydrate adjuvants modulate immune responses through the action of specific innate immune receptors known as lectins that bind sugars. These include the β-glucan receptor, mannan receptor, Dectin-1, toll-like receptors (TLR) and other receptors expressed on monocytes and antigen-presenting cells (APC). Binding of sugars to the relevant innate receptor(s) results in monocyte activation, with nuclear translocation of nuclear factor kappa-B (NFkB) leading to proinflammatory cytokine production that then amplifies the adaptive immune response to a co-administered antigen.

7.4 Alpha-Glucan Adjuvants

Dextran is a branched microbial polysaccharide made up of α-1,6-glucan with α-1,3-branches. Dextran sulphate has marked proinflammatory effects as highlighted by its ability to induce inflammatory colitis in mice. Diethylaminoethyl-dextran (DEAE-dextran) a polycationic derivative of dextran was shown in rhesus monkeys to enhance the antibody response to formalin-inactivated Venezuelan equine encephalomyelitis virus vaccine (Houston et al. 1976). DEAE-dextran similarly enhanced responses to whole cell cholera vaccine in mice (Kaistha et al. 1996). Acetylated dextran (Ac-DEX) microparticles have been used to deliver the TLR7 agonist, imiquimod, to immune cells thereby enhancing TLR7 potency (Bachelder et al. 2010).

7.5 Beta-Glucan Adjuvants

Zymosan consists of β-1,3-glucan protein complexes from yeast cell wall extracts. Zymosan binds to TLR-2 and Dectin-1 on monocytes, thereby activating NFkB. It also activates the alternative complement pathway, contributing to its potent inflammatory action (Schorlemmer et al. 1977; Sato et al. 2003; Dillon et al. 2006). Zymosan also directly activates the inflammasome through ASC and cryopyrin resulting in caspase-1 activation and IL-1β secretion, a feature that it shares with mannan and may be responsible for its high reactogenicity (Lamkanfi et al. 2009). Through these mechanisms zymosan induces nonspecific resistance to bacterial and fungal infection as well as inducing tumouricidal activity in polymorphonuclear cells (Williams et al. 1978; Morikawa et al. 1985; Emod and Joo 1990). When

added to a nasal inactivated influenza vaccine administered to mice, zymosan enhanced the mucosal adjuvant activity of the TLR3 agonist, poly(I:C), through a TLR2-mediated mechanism (Ainai, Ichinohe et al.). Zymosan has also been shown to enhance the response to DNA vaccines through a complement-dependent mechanism (Ara et al. 2001). Oxidised beta-glucan derived from zymosan was able to substitute for Freund's complete adjuvant in induction of collagen-induced arthritis (Hida et al. 2006), consistent with zymosan's proinflammatory effects being sufficiently potent to break immune tolerance. Lentinan is another beta-glucan that is made up of β-1,3-glucan with β-1,6-branches purified from plant sources including shiitake mushrooms (*Lentinus edodes*). Intranasal lentinan induced an enhanced respiratory burst, nitric oxide and IL-6 production by bronchoalveolar macrophages resulting in nonspecific resistance against virus infection (Irinoda et al. 1992). As seen with other immunologically-active polysaccharides, lentinan also exhibits antitumour (Chihara et al. 1987; Jeannin et al. 1988) and antibacterial activities (Drandarska et al. 2005). Addition of lentinan increased the efficacy of a vaccine prepared by transfection of adenovirus-mediated melanoma-associated antigen gene (gp100) into bone marrow-derived dendritic cells for treatment of B16 melanoma in mice, with enhancement of cytotoxic T lymphocytes (CTL) and increased tumour inflammation and necrosis (Wang et al. 2007). Sulphated lentinan enhanced the serum antibody titre and T-cell proliferative response to a Newcastle disease vaccine and reduced mortality of challenged chickens (Guo et al. 2009). Lentinan also increased HIV env-specific Th1 cytokine production and CTL activity to an orally administered recombinant vaccinia virus (rVV) vector expressing gp160 but had no effect on humoral responses (Wierzbicki et al. 2002). Yet another β-1,3-glucan is algal glucan, extracted from *Euglena gracilis*. Algal glucan enhanced humoral and cellular immunity to co-administered herpes virus glycoprotein D peptide antigens and was not toxic, even when administered intravenously at doses up to 25 mg/kg body weight (Mohagheghpour et al. 1995). Beta-glucan particles are purified cell walls of *Saccharomyces cerevisiae* treated so as to remove mannans and yeast proteins, leaving a skeleton primarily made of β-1,3-D-glucans (Huang et al. 2009). Glucan particles bind dendritic cells via the Dectin-1 receptor thereby inducing inflammatory cytokine production. The hollow porous structure of GP allows them to be loaded with antigens including viral vectors resulting in enhanced dendritic cell phagocytosis, upregulation of maturation markers and increased potency on antigen-specific T cells (Huang et al. 2010).

7.6 Mannan Adjuvants

Mannan is a 1,4 linkage polymer of the sugar mannose used as a storage polysaccharide by yeast, bacteria and plants. Binding of mannan by mannan-binding lectin and other C-type lectins of the mannose receptor family leads to complement

activation, opsonisation, phagocytosis, inflammasome activation, caspase 1 activation and inflammatory cytokine production (Takahara et al. 2004; Thiel and Gadjeva 2009; Lamkanfi et al. 2009). The ability of mannan to mature dendritic cells was shown to be mediated through a TLR4-dependent mechanism (Sheng et al. 2006). Mannan and its derivatives including oxidised and reduced mannan have been extensively used to target antigens to dendritic cells, particularly in the area of human cancer vaccines. Mannan when oxidatively coupled to recombinant protein antigen and given intranasally was shown to enhance the production of antigen-specific serum and secretory antibodies (Stambas et al. 2002). A phase 2 clinical study of Muc-1 antigen conjugated to oxidised mannan showed a >4-fold lower rate of tumour recurrence (Vassilaros et al. 2013). Conjugation of myelin basic protein (MBP) to reduced mannan was able to switch the anti-MBP immune response from Th1 to Th2 and protect against experimental allergic encephalomyelitis (Katsara et al. 2009). In a mouse model of Alzheimer's disease, mannan conjugated to Aβ antigen enhanced anti-Aβ antibody production in otherwise hyporesponsive transgenic mice, suggesting an ability of mannan adjuvant to break self-tolerance (Petrushina et al. 2008). Polymannose purified from the *Aloe barbadensis* plant enhanced antibody titres in coxsackievirus B3-immunised mice (Gauntt et al. 2000). Mannose has also been used to target plasmid DNA-containing liposomes to macrophages (Kawakami et al. 2000). Coating of cationic liposomes with mannan significantly enhanced the ability of a DNA vaccine to induce HIV-specific cellular immunity and also the activity of a DNA vaccine against melanoma (Toda et al. 1997; Lu et al. 2007). Mannosylated niosomes have been used as oral DNA vaccine carriers for the induction of mucosal immunity. Niosomes carrying DNA encoding a hepatitis B antigen and composed of Span 60, cholesterol and stearylamine (all coated with the modified polysaccharide O-palmitoyl mannan) were able to induce protective serum titres, cellular immune responses and IgA in mucosal secretions when given orally to mice (Jain et al. 2005). A similar approach was used for topical vaccine delivery using an O-palmitoyl mannan coating to target niosomes to Langerhans cells in the skin (Jain and Vyas 2005). Mannan has similarly been used to enhance delivery of live virus vector vaccines. A recombinant adenovirus vector modified with mannan was used to deliver vascular endothelial cadherin antigen as an antitumour vaccine in mice. Mannan was shown to enhance vaccine responses, resulting in prophylactic and therapeutic inhibition of tumour growth and prolonged survival (Zhao et al. 2011). Similarly, when cDNA of human telomerase reverse transcriptase was inserted into an adenovirus vector and the recombinant adenovirus modified with mannan, the expression of adenovirus in mice was restricted to splenic dendritic cells, consistent with efficient targeting by the surface mannan (Ding et al. 2009). The above data shows that mannan can be used as both an adjuvant and also a dendritic cell-targeting tool for enhanced delivery of live vector vaccines.

7.7 Chitosan Adjuvants

Chitin, a linear β-1-4-linked polymer of D-glucosamine and *N*-acetyl-D-glucosamine extracted from shrimp and chitosan obtained by partial deacetylation of chitin, exhibit a range of immunological effects including macrophage activation and production of inflammatory cytokines resulting in enhanced antibody titres to co-administered antigens (Nishiyama et al. 2006). These effects are mediated by binding of chitin to receptors including Dectin-1, macrophage mannose receptor and TLR-2 (Arca et al. 2009). The addition of chitosan to an intramuscular inactivated influenza vaccine resulted in an increase in antibody titres in mice (Ghendon et al. 2009) and enhanced protection against lethal challenge (Chang et al. 2010). Chitosan particles produced by cross-linking with a counter ion were shown to entrap antigen and enhance its immunogenicity in mice (Prego et al. 2010). By virtue of their mucoadhesive qualities, chitin and its derivatives have been extensively tested as nasal adjuvants. Chitin derivatives such as *N*-trimethyl chitosan chloride enhance the absorption of proteins at mucosal surfaces by inducing transient opening of tight junctions (Kotze et al. 1997). The concomitant use of chitosan microparticles and the mucosal toxin-based adjuvant, LTK63, significantly enhanced the immunogenicity of an intranasal group C meningococcal polysaccharide vaccine (Baudner et al. 2003). Similarly, intranasal alginate-coated chitosan nanoparticles loaded with antigen and CpG adjuvant boosted antibody and cellular responses in mice (Borges et al. 2008). Intranasal plasmid DNA vaccine loaded chitosan nanoparticles induced potent humoral, cellular and mucosal responses (Khatri et al. 2008). In a study with live viral vectors, chitosan improved the immunogenicity in cattle of an intranasal replication-defective adenovirus type 5 vaccine expressing bovine herpesvirus 1 glycoprotein D. The best protection was obtained with vector adjuvanted with glycol-chitosan (Gogev et al. 2004). Similarly, increased immunity was seen when an apathogenic enterotropic live Newcastle disease vaccine was administered by oculonasal route together with chitosan adjuvant to 1-day-old chickens (Rauw et al. 2010). Enhanced protection was also seen with a live virus vaccine against Newcastle disease encapsulated in chitosan nanoparticles (Zhao et al. 2012). Microencapsulation of adenoviral vectors into a chitosan microparticle for mucosal delivery not only protected the virus but also made its release dependent on cell contact (Lameiro et al. 2006). Chitosan also enhanced the protective efficacy of a live attenuated influenza vaccine with the chitosan adjuvant significantly increasing the levels of influenza-specific antibodies and IFN-γ-secreting T cells (Wang et al. 2012). Human phase 1 studies of a chitosan and MPL-adjuvanted intranasal Norwalk virus-like particle vaccine derived from norovirus GI.1 genotype as a mucoadhesive successfully induced high anti-norovirus IgG and IgA titres (El-Kamary et al. 2010), consistent with the ability of chitosan to adjuvant human vaccines. Nevertheless, negative effects of chitosan were observed in cancer vaccine studies. A chitosan-adjuvanted murine adenovirus

cancer vaccine provided minimal protection against tumour challenge, with evidence of reduced antigen-specific CD8+ T cell, IFN-γ and CTL activity (Lemke et al. 2011). This was due to the chitosan inhibiting adenovirus-mediated transgene expression and antigen-presenting cell activation.

7.8 Lipoarabinomannan Adjuvants

Mycobacteria are the major ingredient in Freund's complete adjuvant, which remains the gold standard in terms of adjuvant potency, but also toxicity. Many of the microbial compounds contributing to this adjuvant activity have been progressively identified. Amongst the adjuvant compounds discovered in microbial extracts, many turn out to be carbohydrate-containing structures, including oligosaccharides, glycoproteins and glycolipids with mycobacteria recognised by immune cells through various pathogen-associated molecular pattern (PAMP) receptors, including the TLRs and C-type lectins (e.g. mannose receptor, Dectin-1 and DC-SIGN), with many of these interactions being dependent on carbohydrate structures within the mycobacterial cell wall (Gringhuis et al. 2009). Lipoarabinomannan (LAM) is a major mycobacterial structural cell surface component. LAMs have varying immune activities depending upon their structure. LAMs from nonpathogenic mycobacteria bind TLR2 on macrophages and activate NFkB and induce inflammatory cytokines (Doz et al. 2007). By contrast, mannosylated-LAM from pathogenic *M. tuberculosis* binds to the mannose receptor and DC-SIGN resulting in stimulation of anti-inflammatory cytokines (Doz et al. 2007). Hence, LAMs can be either pro- or anti-inflammatory, depending on their origin, and which innate receptors, e.g. mannose receptor, TLR1/TLR2, TLR4 or DC-SIGN, they signal through, highlighting the subtleties of carbohydrate signalling (Doz et al. 2007). Nevertheless, LAM-derived arabinomannan oligosaccharides from *M. tuberculosis* covalently conjugated to the mycobacterial antigen, Ag85B, protected animals against mycobacterial challenge when administered with Eurocine L3, a monoglyceride adjuvant (Hamasur et al. 2003). No data is available on the use of LAM as an adjuvant for live virus vector vaccines.

7.9 Muramyl Dipeptide Adjuvants

MDP (*N*-acetyl muramyl-L-alanine-D-isoglutamine) was first identified from a mycobacterial peptidoglycan fraction known to have potent adjuvant activity (Yamamura et al. 1976). MDP has been tested on its own and as a component of more complex adjuvant formulations. MDP binds and activates innate immune receptors, including NOD2 (Uehara et al. 2005), and TLR receptors (Takada and Uehara 2006). This leads to potent activation of NFkB, induction of inflammatory cytokines and dendritic cell maturation. The carbohydrate moiety of MDP is critical

to its adjuvant activity as shown by studies in which carbohydrate analogues of MDP were tested in the induction of delayed-type hypersensitivity in guinea pigs (Azuma et al. 1981). This confirmed a structural requirement of the carbohydrate moiety of MDP as only D-mannosamine, D-galactosamine and D-glucose analogues of MDP were active as vaccine adjuvants (Azuma et al. 1981). Many analogues of MDP have been tested as vaccine adjuvants. MTP-PE (*N*-acetyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1,2-dipalmitoyl-sn-glycero-3-(hydroxy-phosphoryloxy)) ethylamide) was included as an immuno-stimulator in the original MF59 squalene emulsion adjuvant (Valensi et al. 1994). However, the MTP-PE component was abandoned because of excessive reactogenicity (Ott et al. 1995). MDP analogues can be made that are either lipophilic or hydrophilic with the lipophilic variants being more immunologically active. When formulated in saline, MDP analogues predominantly stimulate humoral immunity, whereas when incorporated into emulsions or liposomes, they induce cellular immunity. For example, *N*-acetylglucosaminyl-*N*-acetylmuramyl-L-Ala-D-isoGlu-L-Ala-glyceroldipalmitate (DTP-GDP) when formulated as an adjuvant in liposomes induced remission in human metastatic colorectal cancer although it shared MDP's toxicity, producing fever, chills and hypotension at high doses (Vosika et al. 1990, 1991). While the utility of MDP analogues as human adjuvants is restricted by their high reactogenicity, they are found in many veterinary adjuvants. Gerbu adjuvants, for example, are veterinary adjuvants based on GMDP (*N*-acetyl-glucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine), a glycopeptide from the cell wall of *Lactobacillus bulgaricus* (Schwarzkopf and Thiele 1996). Gerbu adjuvants are complex mixtures that may also contain cimetidine, saponin, paraffin, dimethyldi-(stearoylhydroxyethyl)ammonium chloride, mannide monooleate, glycerol, L-proline and ciprofloxacin. MDP was shown to enhance the cellular immune response to a water-in-oil adjuvanted live bovine rotavirus administered intramuscularly to calves (Archambault et al. 1988). Murabutide a less toxic analogue of MDP that has previously been tested in multiple human clinical trials was also shown to act as a mucosal adjuvant and enhanced the immunogenicity of Norwalk virus-like particles administered intranasally to mice (Jackson and Herbst-Kralovetz 2012). The MDP analogue LK415 when tested on chickens immunised with a live vaccine against infectious bursal disease significantly enhanced antibody titres consistent with an adjuvant effect of MDP on live virus vaccines (Rojs et al. 2000).

7.10 Trehalose Dimycolate Adjuvants

Trehalose-6-6-dimycolate (TDM) was previously known as *M. tuberculosis* cord factor and is a potent inducer of inflammatory cytokines with effects including antitumour activity and stimulation of host resistance against infections (Masihi et al. 1983; Sueoka et al. 1995). A number of TDM analogues were synthesised for structure-activity studies, and some attenuation of TDM's toxicity was possible while still retaining adjuvant activity (Fujita et al. 2007). TDM augments both

humoral and cell-mediated immune responses when combined with vaccine antigens, with a comparable efficacy to MDP (Ribi et al. 1975, 1982; Koike et al. 1998). Given its high reactogenicity, TDM is most likely unsuitable for human vaccines but is a component of the long-standing veterinary Ribi Adjuvant System® where it is formulated with squalene oil, monophosphoryl lipid A (MLP) and other components (Ribi et al. 1975, 1982). No data is available on the use of TDM as an adjuvant for live virus vector vaccines.

7.11 Lipopolysaccharide Adjuvants

Bacterial lipopolysaccharide (LPS) is a potent inducer of macrophage activation and inflammatory cytokine production. LPS itself is too toxic to be used as a human adjuvant leading to the development of less reactogenic analogues focusing on the lipid A component that consists of a β-(1,6)-linked D-glucosamine disaccharide phosphorylated at 1-O and 4′-O-positions. In low-acid conditions, lipid A can be hydrolysed to remove the 1-phosphate group, and subsequent mild alkaline treatment leads to removal of the fatty acid at position 3 resulting in monophosphoryl lipid A (MPL). MPL has lower toxicity than LPS but retains immuno-stimulatory activity (Masihi et al. 1986). MPL signals via TLR4 with preferential signalling through the downstream TRIF adaptor rather than MYD88 adaptor, explaining its reduced reactogenicity when compared to LPS (Cekic et al. 2009). MPL has been used in a variety of proprietary adjuvant formulations, including GSK's AS02 and AS04 adjuvants where MPL is used in combination with QS21/oil-in-water emulsion or aluminium hydroxide, respectively. AS04 adjuvant is contained in licensed vaccines against hepatitis B (Fendrix®) and human papilloma virus (Cervarix®) (Tong et al. 2005; Schiller et al. 2008). MPL has also been shown in mice to act as a mucosal adjuvant for influenza virus-like particles with a similar efficacy to alum or CpG adjuvants (Quan et al. 2013). No data is available on the use of MPL as an adjuvant for live virus vector vaccines, which may reflect formulation incompatibility with viruses given the highly hydrophobic nature of MPL.

7.12 Saponin-Based Adjuvants

Saponins from Rhamnaceae, Araliaceae, Polygalaceae and Fabaceae plant families have all been reported to have adjuvant activity (Lacaille-Dubois and Atta ur 2005). The most extensively characterised saponin adjuvant is QS21. QS21 is a saponin derived from Quil A, a mixture of triterpenoid glycosides derived from the bark of the South American soap bark tree, *Quillaja saponaria* (Kensil et al. 1995). QS21 is an acylated saponin at the 4-hydroxyl position on fucose with two linked 3,5 dihydroxy-6-methyloctanoic acids (Kensil et al. 1996). QS21 induces inflammatory cytokines and imparts a Th1 bias in vaccine responses (Kensil et al. 1995; Meraldi

et al. 2005). QS21 has been used in numerous vaccine trials in the cancer field, alone, complexed with cholesterol as ISCOMS (Pham et al. 2006) or mixed with MPL in an oil-in-water emulsion such as GSK's AS02 adjuvant. Because of its ability to lyse cell membranes, adverse reactions including injection site pain and hemolysis can be major limiting factors in use of QS21 (Sun et al. 2009). To overcome these problems a range of chemically modified variants of QS21 have been created. GPI-0100 is a variant with incorporation of the C-12 alkyl chain through a stable amide bind at the carboxyl group of the glucuronic acid residue of deacylated saponin (Marciani et al. 2000; Quenelle et al. 2008). GPI-0100 was shown to be 20 times less lethal in mice than QS21 and stimulates a Th2-like immune response, whereas QS21 stimulates a Th1-like response (Ragupathi et al. 2002). A number of completely synthetic isomers of QS21, called QS21-Xyl and QS21-Api, have been developed (Deng et al. 2008). Other plant-based saponins also have adjuvant activity. For example, glycyrrhizin, a triterpenoid saponin glycoside of glycyrrhizic acid, the main sweet-tasting compound in liquo rice root, activates NFkB and induces inflammatory mediators in murine macrophages and has adjuvant activity when co-administered with antigen (Dai et al. 2001; Raphael and Kuttan 2003). Due to their chemical nature, saponin adjuvants are unsuitable for use as adjuvants with live viral vectors due to their propensity to disrupt cell membranes thereby restricting their use to inactivated or recombinant antigens (Newman et al. 1997) or DNA vaccines (Sasaki et al. 1998).

7.13 Mechanisms of Carbohydrate Adjuvant Action

Many of the sugar-based adjuvants described above including zymosan, mannan, MDP, TDM and LPS work by binding and activating innate immune receptors including TLRs, NOD2 and C-type lectins, resulting in inflammatory cytokine production and thereby enhancement of vaccine immunogenicity (Lee et al. 2001; Nishiyama et al. 2006; Huang et al. 2009). Many carbohydrate adjuvants including MDP, LPS, zymosan, mannan, β-glucan and delta inulin activate complement, and this may also contribute to their adjuvant activity (Ray et al. 1979; Kawasaki et al. 1987; Bohana-Kashtan et al. 2004; Rawal et al. 2009). Chemotaxis induced by IL-8, MCP-1, MIP-1α and MIP-1β, may also play a role in carbohydrate adjuvant action. Phosphomannosyl structures, for example, potently induce lymphocyte migration (Weston and Parish 1991; Dong et al. 2007). Some carbohydrate compounds such as zymosan, mannan and QS21 directly activate the inflammasome, thereby contributing to their adjuvant activity (Lamkanfi et al. 2009). Other adjuvant actions of polysaccharides such as mannan include ability to target antigens directly to dendritic cells. QS21 may have a direct action on T cells as the aldehyde group at C4 of the aglycone unit of QS21 may form a Schiff base with the amino groups of receptors on the T-cell surface thereby providing co-stimulatory signals (Kensil et al. 1996). The odd man out amongst

the carbohydrate adjuvants is delta inulin as it does not activate TLRs, the inflammasome or other innate immune receptors and thereby does not induce inflammatory cytokine production. Nevertheless, it still shares potent adjuvant activity as measured by enhancement of antigen-specific T- and B-cell memory responses. This appears to be through a unique ability to enhance antigen-presenting cell function without activation of inflammatory cytokine genes.

7.14 Conclusions

Sugar structures play a critical role in immune function, and it is not surprising, therefore, that sugar-containing compounds should be a fertile ground for discovery of new immune adjuvants. Carbohydrates are generally safe and well-tolerated, critical attributes for a human adjuvant. Amongst carbohydrates with adjuvant activity, the polysaccharides stand out as promising. The propensity of some polysaccharides such as dextran, zymosan and mannan to potently induce inflammatory cytokines may limit their use in prophylactic human vaccines. Delta inulin is a unique polysaccharide adjuvant that works via TLR- and inflammasome-independent mechanisms and does not induce inflammatory cytokine production. This results in a favourable tolerability and safety profile making it a strong candidate for future human prophylactic vaccine development. As a group, the polysaccharide adjuvants are all compatible for formulation with live virus vector vaccines, and many have positive efficacy data in this context. Given the difficulties of adjuvanting live vector vaccines, this makes polysaccharide adjuvants prime candidates for development in this role.

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