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CURRENT VACCINE ADJUVANTS: AN OVERVIEW OF A DIVERSE CLASS

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1. ABSTRACT

Numerous compounds are under evaluation as immunological adjuvants for improvement of vaccine performance. This review will briefly summarize some of the many diverse substances that are currently being utilized as vaccine adjuvants in preclinical and clinical studies.

2. INTRODUCTION

Modern vaccines, based upon recombinant technology, have the advantage of increased safety, but suffer from decreased immunogenicity compared to the killed pathogen vaccines of the past. Fortunately, this can be corrected by formulation with the appropriate "adjuvant" to enhance immunogenicity. What constitutes an adjuvant? This term covers a broad range of substances. Medzhitov and Janeway note "although adjuvants have a long history, so far they have only been defined operationally as any substance that increases the immunogenicity of admixed antigens." (1) This broad definition of "immunogenicity" has traditionally been defined as an increase in serum antibody response. However, this definition fails to capture all the potential influences of an adjuvant. Some adjuvants promote different $CD4^+$ T helper (Th) cells, either towards Th1, Th2, or a mixed Th1/Th2 response that activate different components of cellular and humoral immunity. Some adjuvants are useful for parenteral immunization only whereas others are useful primarily through mucosal routes of immunization and induce their most important effects on secretory antibody response. Still some adjuvants activate innate immunity and induce cytokine secretion or natural killer (NK) cell activation. The choice of adjuvant or of adjuvant formulation depends upon the needs of the vaccine, whether it be an increase in antibody response in magnitude or in function, in cell mediated immune response, in reduction in antigen dose, or in induction of mucosal immunity. Hence, no one adjuvant is ideally suited for all vaccines. This review will briefly summarize some of the many diverse substances that are currently being utilized as vaccine adjuvants.

A vaccine adjuvant can have many influences on immune responses. The properties of vaccine adjuvants include enhancing the kinetics and persistence of response to antigens, influencing not simply antibody quantity, but also avidity, specificity, isotype, and subclass. Another influence of adjuvants is enhancement of cytotoxic T lymphocyte (CTL) response. This property can be especially important in the development of recombinant antigen vaccines for viral or intracellular organisms. Adjuvants can also increase immune response in immunologically compromised individuals, including immunologically immature (neonates), aged, and immune suppressed individuals. Adjuvants may decrease the amount of antigen required to invoke a protective response,

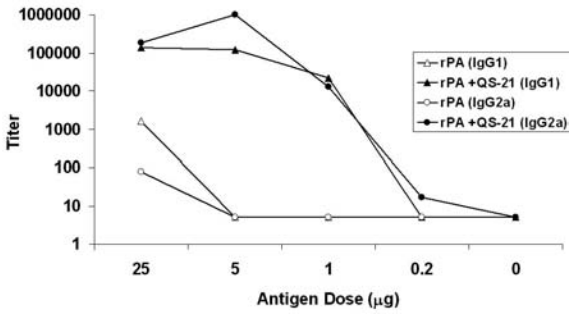

Figure 1. Example of Effect of an Adjuvant on Antigen Dose-Sparing and on Antibody Isotype**.** This illustrates the adjuvant property of antigen dose sparing. C57BL/6 mice were immunized subcutaneously at days 0 and 14 with various doses of recombinant *B. anthracis* protective antigen (PA, obtained as a gift from Dr. Stephen Leppla, NIH) with and without adjuvant QS-21. Serum IgG1 and IgG2a responses specific to the recombinant antigen PA were measured by EIA on serum collected on day 28.

thus potentially reducing other undesirable or unrelated side effects resulting from high doses, reducing cost of the vaccine and/or improving ability for rapid response to vaccine crisis with low antigen supply. Finally, some adjuvants aid mucosal immune responses. Figure 1 illustrates the effect that a strong adjuvant can have on two of the above properties. It shows the significant influence that an adjuvant (QS-21) can have on serum antibody response in mice. The antigen is recombinant anthrax protective antigen, an antigen relevant to current bioterrorism efforts. The dose of antigen is reduced by 25-fold by the inclusion of the adjuvant; this influence is observed on two different types of antibody isotypes, IgG1 (Th2 type) and IgG2a (Th1 type), respectively. This antigen dose-sparing effect on different types of antibody responses could be critical to vaccine crises, such as vaccine development for new strains of influenza virus or new infectious agents like severe acute respiratory syndrome (SARS)- associated coronavirus (SARS-CoV), where antigen production might otherwise be the limiting factor. This is just one example of the critical roles that adjuvants can play in vaccine development.

3. POTENTIAL INFLUENCE OF ADJUVANT ON PATHWAYS OF IMMUNE RESPONSE

An overview of adjuvant effects on immune responses is shown in figure 2. One potential strategy is to use adjuvants that mimic pathogen associated molecular patterns (PAMP) that are capable of binding pattern recognition receptors (PRR) on cells of the innate immune system or adjuvants that resemble "danger signals" recognized by antigen presenting cells (APCs). Their effect is to trigger the recruitment of APCs, release of cytokines and chemokines, and/or upregulation of co-stimulatory molecules. These influence the initial innate immune response, but also influence the antigen specific adaptive immune response through effects on APCs. A second strategy for influencing the adaptive immune response is to use adjuvant "vehicles", i.e. antigen carriers, that prolong antigen exposure to the immune system and /or increased antigen uptake by APCs, thus prolonging T and B cell activation.

Preferentially, an adjuvant formulation employs both strategies – antigen prolongation and stimulation of innate immunity. For example, traditional Freund's complete adjuvant is a water-in-oil emulsion which releases antigen slowly from the site, but also contains an active immunomodulator (muramyl-di-peptide) that binds to PRR on innate immune cells resulting in activation of APCs, providing an optimized immune response. A better understanding of the effect of adjuvants on these signals will lead to the rational design of adjuvants. For example, many small molecule adjuvants were initially identified by their effects on immunogenicity, but are now known to mimic PAMP and to bind to PRR on innate immune cells. Knowledge of specific PRR and their ligands will lead to the ability to design small molecule ligands that have potential for activation of a desirable pathway.

Known PRR on cells of the innate immune system are numerous and are reviewed in Bendelac and Medzhitov (2). These include complement receptors on B cells (activated by microbial carbohydrates) and the invariant T cell receptor (TCR) on NKT cells (activated by the presentation of CD1d-restricted alpha-galactosylceramide [alpha-GalCer], an anti-tumor agent from sea sponges). With respect to developing new adjuvants, significant current discovery efforts have focused on binding ligands for the Toll-like receptor (TLR) family which consists of 9 known receptor molecules. This receptor is a transmembrane protein consisting of extracellular leucine-rich repeat regions and a cytoplasmic domain homologous to the signaling component of the interleukin (IL)-1 receptor (3). Many microbially derived adjuvants (muramyl dipeptide, lipopolysaccharide derivatives, oligodeoxynucleotides containing CpG motifs) are derived from PAMPs which are TLR ligands. Although different TLR molecules share a common MyD88 signaling pathway for activating NF-kappaB transcription factors and subsequent cytokine production, stimulation via different TLRs an lead to different immunological outcomes.(4) For example, ligands of TLR9 (such as bacterial DNA) were noted to be more effective at stimulating antigen-specific CTL responses than ligands of TLR4 (such as LPS) or TLR7 (such as imiquimod), although all induce maturation of dendritic cells (DCs) *in vivo.*

4. ADJUVANT DELIVERY SYSTEMS (ANTIGEN VEHICLES)

Some adjuvants can be considered as antigen delivery systems, and thus are also known as antigen "vehicles". They are designed to combine into a complex with the antigen by way of absorption, co-precipitation, or through encapsulation of the antigen. These adjuvants primarily influence immunogenicity through antigen retention

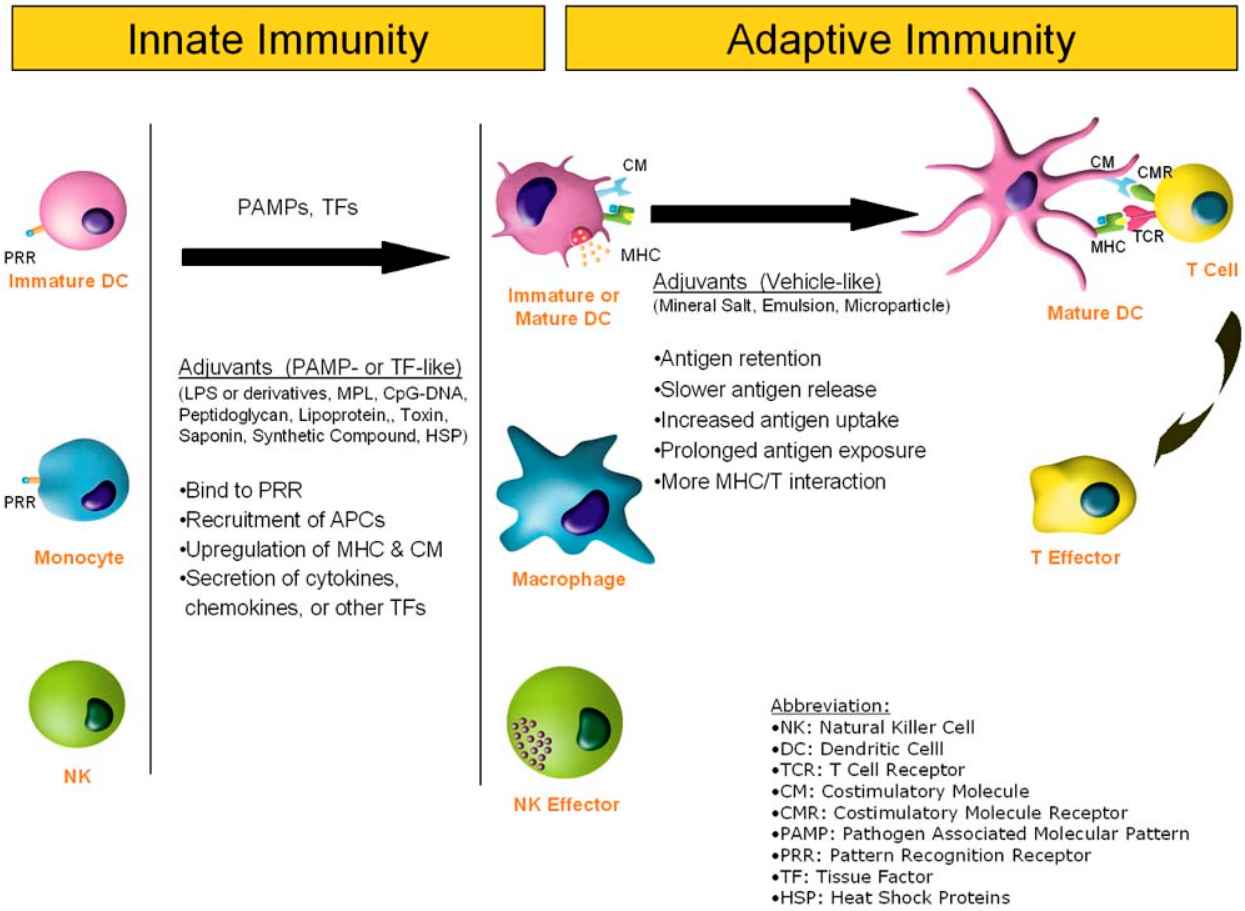

Figure 2. Influence of Adjuvants on Innate and Adaptive Immune System**.** Adjuvants influence both innate immunity and adaptive immunity. Some influence immunity through interaction with pattern recognition receptors associated with cells of the innate immune system; other adjuvants influence antigen persistence and hence prolong antigen presentation.

although other mechanisms have been proposed as well. The adjuvants in Table 1 are a short list of various adjuvant vehicles that have been used in licensed human and veterinary vaccines as well as experimental vaccines.

Antigen delivery systems function primarily by prolonging the presentation of antigen in draining lymph nodes. This can be accomplished by antigen retention at the site of injection, i.e. by forming an antigen "depot" from which antigen can be slowly released *in vivo*, thus ensuring a prolonged exposure to immune cells. The antigen depot theory was first proposed in 1931 (5). Mineral salt adjuvants, emulsions, and microparticles all have the potential to prolong antigen retention. In addition, some formulations also serve to protect the antigen. A second method by which these antigen delivery systems may enhance antigen presentation is by enabling increased uptake by phagocytic cells. This is especially true for antigens in particulate form such as in liposomes or microparticles. These help to mimic the particulate nature of a whole pathogen. The immunological advantage of particulate, as opposed to soluble, formulations is enhanced uptake by DCs and macrophages. The particulate nature of antigens can also heavily influence different endocytic pathways which in turn dictate the patterns of proteolysis and MHC loading for these antigens. In addition, particulate antigen formulations facilitate the formation of opsonins thus enabling higher efficiency in activating B cells through crosslinking of B cell receptors.

The most commonly used antigen vehicles in licensed vaccines are the aluminum salt adjuvants (aluminum hydroxide or aluminum phosphate). These are either co-precipitated with antigen or are used as a pre-formed gels (eg. Alhydrogel, Adju-Phos) for adsorption of antigen (6). The aluminum adjuvants are noted for enhancing primarily a humoral, Th2-biased, immune response.

Emulsion adjuvants, a dispersion of an aqueous antigen in an immiscible media, oil, stabilized by surfactants, have also been utilized in vaccines. Incomplete Freund's adjuvant (IFA), an example of a water-in-oil adjuvant based upon mineral oil, was used as an adjuvant for experimental human vaccines up until 1945, but was discontinued due to reactogenicity (sterile abcesses or cysts, related to the quality of the surfactant or the oil) and potential carcinogenicity related to impurities in the Aracel

Table 1. Antigen Delivery Systems (Vehicles)

TYPE	SUBTYPE	EXAMPLES
Emulsions	Water-in-Oil	Montanide ISA 720 and 51 (7)
	Oil-in-Water	MF59 (8)
Mineral Salts	Aluminum	Aluminum Hydroxide; Aluminum Phosphate gel or suspension (130)
	Calcium	Calcium Phosphate gel or suspension (131)
		Calcium phosphate nanoparticles (132)
Particulate	Microparticles	Poly(lactide-coglycolide) (PLG) (133, 134)
		Poly(lactic-coglycolic acid) (PLGA) (135)
		Chitosan (13)
	Liposomes	Virosomes (14)
	ISCOM	Antigen-containing ISCOMs [1] (15)

[1] ISCOM= Immune Stimulating Complex

A surfactant. Modern versions of water-in-oil emulsions include Montanide adjuvants, based upon more refined oils and surfactants compared to the traditional IFA. Montanide adjuvants ISA51 (based upon a blend of mineral oil and surfactant mannide monooleate) and ISA 720 (based upon a blend of a vegetable oil and mannide monooleate) have been tested extensively in experimental vaccines, with Phase I and II testing in HIV, malaria, and cancer vaccines (7). In addition to water-in-oil adjuvants, oil-in-water adjuvants have also been developed. An example of an oil-in-water adjuvant is MF59 (8), an emulsion with high stability and small droplet size that enhances primarily antibody and Th2-type cytokine responses (9).

Other antigen delivery systems are particulates that encapsulate antigen. These include microparticles and liposomes. Microparticles such as those prepared from poly (lactide-co-glycolide) (i.e. PLG) or poly(lactic-co-glycolic acid) (i.e. PLGA) are intended to be biodegradable and can be manufactured for different desired properties. For example, slower antigen release is associated with higher molecular weight polymers and/or a high lactic acid to glycolic acid ratio in the polymer backbone (10). Microparticles for antigen encapsulation have also been prepared from other biodegradable substances such as poly(methyl methacrylate) (11, 12) or chitosan, a linear polysaccharide (13). Other particulates are designed to resemble viruses. One prominent example is the virosomal vaccine. A virosome vaccine is prepared from reconstituted viral glycoproteins into a membranous (liposomal) structure such that the resulting vaccine has a similar spatial relationship of viral spike proteins on the surface so as to resemble the virus, and has a particle size which is similar to the virus (approximately 80-120 nm, (14)). The virosome adjuvant system has been applied to influenza vaccine development; currently a virosomal influenza vaccine is licensed in Europe as Inflexal V®. Still another particulate adjuvant is the Immunostimulating Complex, also known as ISCOM (15). Like the virosome vaccine, ISCOM consists of lipids and viral glycoprotein, but also contains an active immunomodulator (saponins derived from *Quillaja saponaria*). ISCOM particles have a unique cage-like structure, with ring-like units about 12 nm in diameter. ISCOMs have been evaluated with a wide variety of viral proteins, including influenza (16) and measles glycoproteins (17). ISCOMs prepared with the partially purified saponin ISCOPREP 703 have been noted to induce both Th1 and Th2 immune responses (18). ISCOM are not limited to antigens with membrane-binding domains. Soluble antigens can be incorporated into the ISCOM particle by covalent attachment of fatty acid (19) or by covalent conjugation to a preformed ISCOM particle (20). Other studies have focused on encapsulation of antigen into liposomes. Particle size and the ability to be phagocytosed by macrophages may be important to the induction of Th1 or Th2 responses by liposomes, perhaps by influencing the initial antigen uptake, processing and presentation. Antigen-containing liposomes greater than 225 nm in diameter tended to induce Th1 cytokines whereas liposomes less than 155 nm induced Th2-cytokines (21). Liposomes that are pH sensitive and release contents into the cytosol after fusion with the endosomal membrane have also been shown to enhance CTL and Th1 responses (22).

Some emulsion type adjuvants may not necessarily function by affecting antigen retention through formation of an antigen depot, but instead may be treated as particulate. For example, the small droplet size adjuvant MF59, although in the emulsion category, does not promote an antigen depot (established through studies of radiolabeled antigen (23)). MF59 does cause an influx of macrophages to the site of injection that take up the MF59-antigen formulation, delivering it to the draining lymph node where the antigen and MF59 is acquired by resident DCs from apoptotic macrophage (24).

5. IMMUNOMODULATORS

Immunomodulator adjuvants are typically small molecules that exert their adjuvant functions through mechanisms other than antigen retention, typically through direct stimulation of innate immune cells such as monocytes, macrophages, NK cells, NKT cells, and DCs. Examples of immunomodulators are the active component of Freund's complete adjuvant as well as lipopolysaccharide. It is now known that many classic immunomodulators exert their first effect on the innate immune system, with subsequent effect of those signals on the adaptive immune system. Many immunomodulators are PAMPs that interact with PRR, providing the signal required for activation of quiescent APCs expressing these receptors. Various immunomodulators are presented in table 2. Many immunomodulators are derived from pathogens. Others may mimic pathogen structures. Some of the more common immunomodulators will be described in this section.

Table 2. Immunomodulators

TYPE	SUBTYPE	EXAMPLES
Bacterial structures (natural, semisynthetic, or synthetic)	Lipid A or derivative	MPL semisynthetic from lipid A of *Salmonella minnesota* (26)
		RC- 529 synthetic aminoalkyl glucosaminide 4-phosphates (26)
		OM174 (29, 31)
	Peptidoglycan	N-acetyl muramyl-L-alanyl-D-isoglutamine (34)
		MTP-PE (35)
	Lipoproteins or Lipopeptides	19-kDa mycobacterial lipoprotein (37, 41)
		Pam_3CSS (synthetic lipopeptide covalently attached to antigen) (38, 39)
	Synthetic oligodeoxy-nucleotides (ODN) containing unmethylated CpG motifs	CpG-B (aka K-ODN) (43, 136, 137)
		CpG-A (aka D-ODN) (53, 138)
	Enterotoxins	Cholera Toxin B subunit (62)
		Heat labile enterotoxin (LT) mutants LTK63 and LTR72 (58, 60, 61)
Plant products	Saponins	QS-21 Purified triterpene glycoside (68)
		Iscoprep 7.0.3 Partially purified triterpene glycoside (69)
	Alkaloid glycoside	Tomatadine (75)
Marine Products	Glycosylceramide	Alpha-galactosylceramide (139, 140)
Antiviral Agents	Imidazoquinoline	Imiquimod; Resiquimod (49, 77)
Endogenous Cell Products	HSPs	HSP70 [1] (106)
		gp96 (106)

[1] HSP = Heat Shock Protein

5.1. Lipopolysaccharide derivatives

Lipopolysaccharide (LPS) from Gram-negative bacteria is a stimulator of the innate immune response, binding to TLR4 on APCs, initiating activation of APCs and a cascade of cytokine release that is critical for its adjuvant function. However, it also has endotoxic action. The lipid portion of LPS is known as lipid A and consists of a diglucosamine saccharide that is phosphorylated at positions 1' and 4'. The diglucosamine backbone is acylated with 6-7 acyl chains in amide or ester linkage. Lipid A retains the immunological effects of LPS, but is still highly toxic. Although some effort has been made to reduce lipid A toxicity through incorporation into liposomes followed by adsorption onto aluminum hydroxide (25), more effort has recently been focused on derivatizing lipid A to reduce its toxicity and to retain its ligand function for TLR4 and associated adjuvant function.

The most advanced lipid A derivative is monophosphoryl lipid A (MPL), which has been tested extensively in human clinical studies (26). MPL is derived from *Salmonella minnesota* strain R595 LPS. It is modified to contain a single phosphate group rather than the double phosphate group in the parent lipid A. It retains the hexa-acyl component of lipid A (27). As MPL is derived from a natural source, this hexa-acyl component is somewhat heterogeneous although the different species are closely related in degree and type of acylation. The removal of a phosphate group significantly detoxifies lipid A (28). However, MPL retains ligand function for TLR4. Its adjuvant function consists of stimulation of macrophage and DC maturation. In addition, it triggers a cytokine and chemokine cascade. Further improvements have been made in the area of synthetic lipid A mimetics, known collectively as aminoalkylglucosaminide 4-phosphates (AGPs). AGPs are mimetics of the hexa-acyl component present in MPL. These have been shown to be useful for structure/activity relationship studies, including modifications of acyl chain length and spacing. One AGP (RC-529) has similar properties to MPL and has advanced to clinical testing (26). Both MPL and AGP RC-529 stimulate innate immune responses.

Another lipid A derivative is OM-174. This is a water soluble tri-acyl lipid A from *E. coli*, prepared by a combination of alkaline-catalyzed hydrolysis to remove ester-linked fatty acids and acid hydrolysis to remove O-polysaccharide and core oligosaccharide regions of LPS (29). OM-174 is reduced in endotoxic activity by 10^5 fold, compared to *E. coli* LPS, but it retains much of the function for stimulating cytokines. In addition, it retains activity for inducing maturation of murine DCs and their migration into the T cell zone of draining lymph nodes *in vivo* (30). Although initially developed as an anti-tumor agent, it has been shown to be useful as a murine adjuvant when used with a synthetic peptide from *Plasmodium berghei* circumsporozoite protein (31). It was shown to enhance antibody titers to peptide as well as an improved $CD8^+$ T cell response (measured by interferon (IFN)-gamma Elispot).

5.2. Peptidoglycans

The classic Freund's adjuvant consists of a water-in-oil emulsion containing dried tubercle bacilli (32). The bacilli were shown to contain an active immunomodulator that was also responsible for local and systemic lesions (33). The active component was later shown to be a peptidoglycan consisting of a disaccharide-tetrapeptide. The minimal active unit is an N-acetyl muramyl-L-alanyl-D-isoglutamine (also known as MDP) (34). This synthetic

peptidoglycan could be substituted for the tubercle bacilli in Freund's adjuvant. A derivative peptidoglycan (35), in which a fatty acid was introduced to reduce MDP's pyrogenicity [MDP-L-alanine-(1',2'-dipalmitoy-sn-glycero-3'-hydroxyphosphoryloxy)-ethylamide (MTP-PE)], was evaluated in clinical trials in combination with the oil-in-water emulsion MF59. Unfortunately, this immunomodulator contributed to increased systemic toxicity in a flu vaccine trial (36).

5.3. Lipoproteins

Bacterial lipoproteins can have potent effects on innate immune responses. An example is the native mycobacterial 19-kDa lipoprotein, which is acylated at an N-terminal cysteine and induces monocytes to secrete IL-12 to levels similar to that induced by LPS (37). This natural lipoprotein and lipopeptide mimetics such as PAM_3Cys_3 (P_3C_3) coupled to antigen (38, 39) both act through a heterodimer of TLR2 and TLR1 (40, 41). A structure function analysis of various synthetic lipopeptide derivates indicated that P_3CSK_4 (N-Palmitoyl–S–[2, 3-bis(palmitoyloxy)-(2RS)-propyl]–(R)–cysteinyl–lysyl-lysyl-lysyl-lysyl-lysine) was highly active in stimulating macrophages for nitrous oxide release, for NF-kappaB translocation, and for tyrosine protein phosphorylation as well as improving immune response to genetic immunization when mixed with DNA (42).

5.4. CpG

Another class of adjuvants derived from bacteria are CpGs, based upon sequences in bacterial DNA. Unlike mammalian DNA, *E. coli* and other bacterial DNAs are unmethylated. In addition, the CpG dinucleotide is rare in mammalian DNA compared to bacterial DNA. The term CpG refers not to a single species of a given sequence but to the class of molecules consisting of bacterial DNA or oligodeoxynucleotide (ODN) sequences containing unmethylated CpG dinucleotides. These unmethylated CpG dinucleotides, in particular base sequence contexts (termed CpG motifs), were shown to be potent Th1 immunomodulators (43-45). The activity was noted to be different than that stimulated by LPS (46). *E. coli* DNA was shown to be a potent immunomodulator, inducing IL-6 synthesis from monocytes. CpG ODN is a potent stimulator of innate immune responses, including NK cell activation, IFN-gamma and IL-12 cytokine production. In fact, certain CpG ODN sequences have been shown to protect against pathogen challenge when used in the absence of antigen (47, 48).

Bacterial DNA and CpG (ODN) were shown to bind to the PRR, TLR9 (49). This receptor confers species specificity to CpG. The optimal CpG motif for activation of human cells is different from the optimal CpG motif for activation of murine cells. This was found to be due to recognition of mouse-active CpG sequences by the murine TLR9 and recognition of human-active CpG sequences by human TLR9 (50).

There are at least two structurally distinct classes of CpG ODN with different biologic activities. "K"-ODN (also known as "B"-ODN) contain phosphorothioate backbones and contain multiple TCGTT and/or TCGTA CpG motifs. The "K"-ODN induce the maturation of plasmacytoid DC and trigger monocytes and B cells to proliferate and secrete IL-6 and IgM (51). A leading example of "K" (or "B")-ODN is the CpG known as 2006, which is currently in Phase I human clinical trials of hepatitis B and flu vaccines (status reviewed by Klinman (52)). "D"-ODN (also known as "A"-ODN) typically contain mixed phosphodiester/phosphothioate backbones. The "D"-ODN contain a single hexameric purine / pyrimidine / CG / purine / pyrimidine motif flanked by self-complementary bases that form a stem-loop structure capped at the 3' end by a poly G tail. These "D"-ODN induce APC maturation and secretion of IFN-alpha and IFN-gamma (53).

5.5. Enterotoxins

Bacterial enterotoxins are also potent adjuvants. These have been found to be particularly useful as mucosal (administered via intranasal or oral route) adjuvants. The best known enterotoxin adjuvants are derived from *Vibro cholerae* (cholera toxin, CT (54)) and from *E. coli* (heat-labile enterotoxin LT (55)). In unmodified form, these are highly toxic and result in accumulation of intestinal fluid and diarrhea (56). Despite this significant toxicity, oral administration of these adjuvants results in induction of serum IgG and mucosal IgA to co-administered antigens. The type of immune response associated with oral CT administration is primarily a Th2 response (57).

The desirable mucosal adjuvant activity associated with the enterotoxins has led to an effort to detoxify the toxins by removal or genetic mutation of the A subunit. The A subunit is responsible for toxicity through its enzymatic activity and subsequent promotion of cAMP accumulation in intestinal epithelial cells. The pentameric "B" subunit of both LT and CT binds GM1 on cell membrane surface receptors and aids in the transfer of the A subunit into the cytoplasm of the cell. Although non-toxic, the B subunit is a relatively poor adjuvant compared to the holotoxin. For example, the cholera toxin B subunit is largely inactive when admixed with antigens, although it may be effective when linked to antigens (54). The search for an LT or CT based adjuvant has focused on reduction of toxicity associated with the "A" subunit without complete elimination of "A" subunit enzymatic activity, since that enzymatic activity may be related to some aspects of LT adjuvant function. Two mutants, LTK63 and LTR72, were generated by site-directed mutagenesis of the "A" subunit to eliminate or reduce enzymatic activity (58). LTK63, containing a substitution of serine 63 with lysine, is enzymatically inactive (59). LTR72, containing a substitution Ala→Arg in position 72 of the "A" subunit, retains some (0.6%) of the wild type LT enzymatic activity, and is 10^5 fold less toxic in an *in vitro* assay on Y1 cells (60). In contrast, LTK63 completely lacks enzymatic activity and has no apparent toxicity (61). The mutant with greater A subunit enzymatic activity (LTR72) also has greater mucosal adjuvanticity compared to LTK63, in which the enzymatic activity is entirely eliminated. This has been postulated to be through binding to GM1 on APCs, subsequent activation of the adenylcyclase system

Table 3. QS-21 Stimulation of Innate Immune Response

TREATMENT	MURINE NATURAL KILLER CELL ACTIVITY [1] (% LYSIS)		CYTOKINE AND CHEMOKINE RELEASE FROM MURINE MACROPHAGES [2] (PG/ML)		
	Spleen	**Liver**	**TNF-alpha**	**IL-6**	**MIP-1alpha**
No Adjuvant	11.1%	22.1%	240	75	5800
+ QS-21	22.4%	57.8%	5390	200	30,000

[1] Balb/c mice (female, 8-10 weeks of age) were injected subcutaneously with 200microliters of saline or 10 micrograms QS-21 in saline on days 1 and 2. On day 3, splenocytes and liver cells were evaluated for lysis of ^{51}Cr-loaded YAC1 cells (effector:target ratio of 200:1) during a four hour incubation at 37 C. [2] Murine macrophages (RAW 264.7) were cultured in the presence of media or 4 micrograms/microliters QS-21 in media. Cytokines and chemokine levels (TNF-alpha, IL-6, and MIP-1alpha) were measured by EIA on supernatants collected after 24 hours of incubation.

and increased levels of intracellular cAMP, which in turn may influence NF-kappaB transcription factors and cytokine production (62). Hence, development of these mutant enterotoxins has focused on reducing or eliminating toxicity while retaining sufficient immunomodulator activity. LTR72 and LTK63 were compared as adjuvants for Th1/Th2 response. LTR72 appeared to suppress Th1 responses and to enhance Th2 responses whereas LTK63 enhanced Th1; this was related to the absence of cAMP production in APC exposed to the enzymatically inactive LTK63 (63). LT-induced intracellular cAMP appeared to correlate inversely with NF-kappaB expression in murine macrophages.

LT mutants have undergone extensive non-clinical safety evaluation to establish their suitability as potential clinical adjuvants for intranasal vaccines (64). The extensive evaluation was in part due to the observation that a licensed virosomal influenza vaccine containing a natural variant of LT was associated with rare cases of Bell's palsy. This vaccine was withdrawn from the market and non-clinical safety studies were performed in animal models of Bell's palsy. Those studies did not support a link between the LT-containing vaccine and Bell's palsy (65). A second reason for this evaluation was the observation that intranasally administered radio-iodinated CT and CTB adjuvants and vaccine antigens could penetrate the olfactory central nervous system through the olfactory bulbs (66). Hence, this was evaluated using clinical grade of LTK63 and LTR72; however, no such uptake was observed with the clinical batches (64). LTK63 has since advanced to the stage of clinical evaluation in an influenza vaccine.

5.6. Saponins

Plants are an abundant source of immunologically active compounds. Among the most prominent of these compounds are complex triterpene glycosides, also known as "saponins". Saponins derived from the bark of the South American tree *Quillaja saponaria* have significant adjuvant activity. An enriched saponin fraction (QuilA), consisting of greater than 20 structurally diverse saponins, is used in veterinary vaccines (67). Some of these saponins were found to be highly toxic (68). However, a *Quillaja* saponin known as QS-21 was purified to near homogeneity and shown to have potent adjuvant activity and to have an acceptable toxicity, enabling evaluation in human clinical studies (68). Another *Quillaja* saponin preparation (ISCOprep 7.0.3) consists of two partially purified fractions from *Quillaja* (consisting of a mixture of 3 parts of fraction QH-A to 7 parts QH-C) and is depleted of toxic saponins present in intermediate fraction QH-B (69). ISCOMs prepared from ISCOprep 7.0.3 have entered clinical trial evaluation (70).

Characteristics of QS-21 as an adjuvant include strong enhancement of both Th1 and Th2 immune responses in murine models (71). It has been noted to induce CTL responses to subunit antigens (72). Its primary use is an immunological adjuvant for subunit vaccines administered by parenteral routes, but QS-21 has also been utilized by mucosal routes in mice, enhancing both serum and mucosal antibody responses when used orally with tetanus toxoid antigen (73) or intranasally with HIV env DNA (74). Interestingly, QS-21 has some properties of stimulating innate immune responses. It stimulates NK cell activity *in vivo* (murine studies) and cytokine/chemokine release from macrophages *in vitro* (Table 3). A PRR for QS-21 or other saponins in ISCOprep 7.0.3 has not been identified at the present time.

Another plant-derived adjuvant is tomatidine, an alkaloid glycoside derived from the leaves of the wild tomato *Lycopersicon pimpinellifolium* (75). Tomatidine was shown to induce CTL responses in mice to a 9-mer peptide from *Plasmodium berghei* circumsporozoite protein (75) and to ovalbumin (76).

5.7. Synthetic adjuvants

In addition to the pathogen derived and natural products described above, certain synthetic compounds are potent immunomodulators. Among these are the antiviral compounds R-837 (imiquimod) and R-848 (resiquimod). These belong to the imidazoquinoline class of compounds. Although originally identified as antiviral agents, they also have adjuvant activity. Imiquimod was used as an adjuvant for an experimental HSV-2 glycoprotein vaccine in guinea pigs (77). Imiquimod and resiquimod activate immune cells via the TLR7-MyD88-dependent signaling pathway (49). One can assume that the imidazoquinolines resemble a PAMP on a pathogen; however, that pathogen is unknown at present.

5.8. Recombinant cytokines and growth factors

Recombinant cytokines and growth factors have also been evaluated as vaccine adjuvants. Although technically part of the normal immune response, cytokines, administered as recombinant proteins or provided in plasmid or viral vectors for *in vivo* expression, can improve antigen presentation. The primary cytokines and growth factors being evaluated as clinical immunological adjuvants

Table 4. Adjuvant Effects of Cytokines and Growth Factors

CYTOKINE / GROWTH FACTOR	BIOLOGICAL ACTIVITY	VACCINE USE
GM-CSF	Hematopoetic factor. Stimulates production and activation of dendritic cells. Enhanced Antigen Presentation.	Preclinical Studies: HIV env subunit vaccine + GM-CSF (79) HIV env DNA vaccine + plasmid GM-CSF(80) Clinical Studies: Tyrosinase Peptide + GM-CSF; Adjuvant effect noted (86) Hepatitis B Vaccine + GM-CSF; No Adjuvant effect observed (87)
IL-12	Natural Killer cell stimulatory factor, a heterodimer consisting of 40-kDA and 35-kDA subunit. (141) Highly species specific(142) Primes for Th1 and Th2 responses (143)	Preclinical Studies: Leishmania vaccine (81) Mucosal Influenza Vaccine (82) Mucosal Tetanus Toxoid Vaccine (83) Clinical Studies: Phase II study (toxicity observed (88)) 23-valent pneumococcal vaccine (no adjuvant effect observed (89)) Melanoma/influenza vaccine (no adjuvant effect observed (90))
Flt3 Ligand	Ligand for fms-like tyrosine kinase receptor Flt3. Hematopoetic dendritic cell growth factor. Dramatic effects on dendritic cell expansion (84)	Preclinical Studies: Use of human flt3 ligand to expand dendritic cells in mice (84) Adjuvant effect with ovalbumin in mice. Enhanced IgG2a and interferon-gamma secreting antigen-specific T-cells (85) Clinical Studies: Pilot dose escalation Phase I study in human volunteers; enhanced dendritic cell expansion (144). Two trials of Her-2/neu peptide vaccines (MHC-II (91)or MHC-I (92) epitope) + flt3 ligand in subjects with Her-2/neu overexpressing cancer; no peptide specific T-cell responses (91, 92) except for interferon-gamma secreting peptide-specific T-cells (91). Hepatitis B vaccine + flt3 ligand (separate sites). Enhanced DC expansion. No effect of flt3 ligand on antibody titers (93).

have been reviewed by Villinger (78). These include IFN-alpha, beta, and gamma, GM-CSF, IL-2, IL-12, and flt3 ligand. Table 4 reviews some preclinical and clinical studies with cytokines as experimental adjuvants. While cytokines have shown good results in preclinical studies (79-85), there has been mixed success with them as adjuvants in clinical studies (86-93).

5.9. Heat shock proteins (HSPs)

It has been noted that HSPs, intracellular proteins, released by necrotic or stressed cells can activate DCs. Immature DCs phagocytose both necrotic (freeze-thawed) and apoptotic (UV-triggered) cells (94). Necrotic cells were able to activate resting DC whereas healthy cells or those undergoing apoptotic death did not. (94-96) Mice immunized with ovalbumin and freeze-thawed fibroblasts or smashed blood vessels primed T cells to a higher extent than ovalbumin and apoptotic fibroblasts (determined as a DTH response (95)). These DC-activating endogenous signals were termed “danger signals”. The most studied “danger signals” have been HSPs, a class of proteins upregulated when cells are stressed. Basu *et al*. showed that necrotic, but not apoptotic cells release HSPs including Hsp70, Hsp90, calreticulin, and gp96 (96). Murine Hsp70 (cytosolic), Hsp90 (cytosolic) and gp96 (located in the endoplasmic reticulum) were isolated, purified away from contaminating LPS, and shown to stimulate murine macrophages to secrete cytokines which included TNF-alpha, IL-12, and IL-1beta in a dose-dependent fashion whereas control proteins such as ovalbumin or insulin did not (96). In the same study, HSPs were shown to aid in DC maturation and to activate translocation of NK-kappaB. Hence, similar to exogenous PAMP, endogenous HSPs aid in the mediation of innate immune responses, presumably through interaction with PRRs. Receptors identified for various HSPs on APCs include CD91 (97, 98), Toll like receptors (99, 100), and CD40 (100).

Prior to the discovery that HSPs modulate innate immune responses, it was well known that HSPs also serve to chaperone nascent polypeptides and natural peptides (101). Much research has focused on the chaperone role of

HSPs to aid in the uptake of antigen by APCs, hence aiding in the development of adaptive immunity. In particular, the heat shock protein-peptide complex is believed to enable the introduction of antigen into the MHC class I antigen presenting pathway of DCs and macrophages and hence to stimulate $CD8^+$ T cell responses (97, 102). Due to their activity to stimulate $CD8^+$ T cell responses, HSPs derived from tumors (and hence bearing tumor specific antigenic peptides) have been shown to be useful in tumor rejection models in mice, including fibrosarcoma, hepatoma, colon carcinoma, melanoma, lung carcinoma, lymphoma, and prostate cancer (103). Autologous vaccines based upon patient tumor-derived HSPs are in clinical development for a number of cancer applications (104, 105). Studies are also underway to evaluate HSPs as carriers for exogenous peptides taking advantage of the heat shock protein as both an immunomodulator and as an antigen carrier. The reconstitution and immunogenicity of exogenous peptides with gp96 (noncovalent complexes) was demonstrated by Blachere (106). Hsp70 or gp96 complexes with various peptides containing MHC class I epitopes were shown to prime mice for $CD8^+$ T cell responses (106). In a variation of that approach, fusion proteins consisting of Hsp70 and various peptides for HIV, human papilloma virus and other diseases have been tested in animals and shown to induce $CD8^+$ T cell responses (107-109).

6. ADJUVANT FORMULATIONS

The optimum adjuvant may often consist of an antigen delivery system that prolongs or improves antigen presentation in lymph nodes and with immunomodulator adjuvants that stimulate recruitment of APCs, release of cytokines and chemokines, and/or upregulation of costimulatory molecules. The classic example is Freund's complete adjuvant, consisting of the water-in-oil incomplete Freund's adjuvant to provide a slow release of antigen plus an active immunomodulator (muramyl-dipeptide). Although not acceptable for human use due to unacceptable side effects (110), Freund's complete adjuvant has, in animal studies, stood for decades as a benchmark for both humoral and cellular immune responses (32).

An example of adjuvant formulation is the combination of aluminum hydroxide, an antigen vehicle, with various immunomodulators. Aluminum hydroxide has a Th2 profile and does not induce cell-mediated immune responses. The Th1/Th2 immunomodulator adjuvant QS-21 was added to aluminum hydroxide-adsorbed proteins; QS-21 induced CTL responses to aluminum-hydroxide adsorbed ovalbumin similar to if QS-21 was added to soluble ovalbumin (72). Similarly, when CpG was added to aluminum hydroxide, hepatitis B antigen-specific antibody titers were significantly higher than the separate adjuvants (45). The efficacy of murine IL-12 in malaria vaccines was improved by adsorption of the IL-12 cytokine onto Alum (111).

Liposomes, emulsions, and microparticles are common carriers for hydrophobic immunomodulators. One example of a complex emulsion-based adjuvant formulation is AS02, which consists of an oil-in-water emulsion with two immunomodulators, monophosphoryl lipid A and QS-21. This formulation was found to be more protective against a malaria challenge in a laboratory setting in a clinical study than the simpler oil-in-water formulations and MPL/Alum (112).

7. ADJUVANT IDENTIFICATION AND EVALUATION

How does one identify an adjuvant? The ability of a substance to augment an increase in immunogenicity of a vaccine defines it as an adjuvant. The traditional starting point for scientists involved in vaccine adjuvant discovery research has been to evaluate substances for increasing antibody titer to a given antigen in an animal model. More recently, this has been broadened to include immunogenicity measurements such as increase in specific antibody isotypes, in cell mediated immune responses such as CTL or IFN-gamma Elispot responses, Th1 and Th2 cytokine responses as well as to provoke a response of the innate immune system including activation and differentiation of DCs. These functional assays enable the qualitative and quantitative profiles of an adjuvant to be defined.

In addition to these assays, understanding of PRR-PAMP interactions enables more cell-based discovery and screening methods for immunomodulator compounds, a high throughput complement to animal studies. An example includes studies of lipopeptides, which are known to bind to TLR2 on macrophages. This understanding of the PRR for the class of compounds enabled evaluation of a broad array of derivatives at different concentrations for nitrous oxide release from macrophage (42). The most potent of these compounds, P_3CSK_4, causes NF-kappaB p65 translocation and enhanced tyrosine protein phosphorylation, confirming the involvement of the TLR – NF-kappaB translocation pathway. Specific genes that were induced or repressed by P_3CSK_4 treatment of bone marrow derived macrophages were examined in a cDNA expression array that monitored 140 genes. Activation of genes for IL-1beta, IL-7, IL-15, G-CSF, CSF-1/M-CSF, CD40L receptor, CD3, and macrophage inflammatory proteins (MIP)-1beta and –2alpha were noted. The results were consistent with activation of the NF-kappaB translocation pathway. In another example of the use of gene expression array analysis, the effect of imiquimod derivative S-28463 on macrophages (113) was examined. From the 588 screened genes, thirteen genes were induced which are known to be involved in macrophage activation and inflammation and included IL-1beta, MIP-1alpha, MIP-1beta, and MIP-2alpha. Hence the use of such tools as gene activation may provide a more precise characterization of the cellular action of adjuvants. However, at present it remains difficult to correlate these results with immunological outcome *in vivo*. The effects of the well known adjuvants aluminum phosphate (predominantly Th2) and Freund's complete adjuvant (Th1/Th2) on gene expression was examined in splenocytes from mice immunized two weeks earlier with toxoid antigens (114). These adjuvants were found to activate 61 genes, with primary differences in the kinetics of the responses, where aluminum phosphate was shown to activate the genes earlier.

Table 5. Clinical Experience with Experimental Vaccine Adjuvants

ADJUVANT	VACCINE
MPL	HSV (117), Melanoma "Melacine" (118)
MF59	Influenza (119), HSV type 2 (120) , Hepatitis B (121) ,HIV (122)
QS-21	Cancer (86), HIV (123), Malaria (124)
AS02	Malaria Phase II (125), HIV (126), Therapeutic Hepatitis B (126)
CpG	Hepatitis B (52), Influenza (52)
ISA 151 and 720	Cancer (7), Malaria (7)

Genes involved in immune function, in apoptosis, and signal transduction were identified. Other than kinetics, clear-cut differences in gene expression did not reveal the underlying mechanisms that lead to the differences between the Th2-inducing aluminum phosphate and the Th1 effects of Freund's complete adjuvant. Hence, it will still be necessary to validate the results from gene expression studies with the outcome from *in vivo* studies of adjuvant function.

8. ADJUVANTS TESTED IN HUMAN STUDIES

8.1. Preclinical toxicological analysis

Many of the adjuvants or formulations described in this review, having been evaluated in pre-clinical safety studies, have advanced into clinical trials. Real and theoretical risks of vaccine adjuvants have been summarized by Kenney and Edelman (115). These include local acute reactions such as abscesses, nodules, ulcers, flu-like illness, anaphylactic reactions, systemic clinical toxicity, induction of hypersensitivity to host tissues resulting in autoimmune arthritis, amyloidosis, or anterior uveitis, glomerulonephritis or meningoencephalitis due to cross-reaction with human antigens, sensitization to tuberculin antigens, immune suppression, carcinogenesis, teratogenesis, or abortigenesis. Pre-clinical and non-clinical safety assessment of adjuvants has typically been carried out in rodents (mice, rats and rabbits), following guidelines developed by regulatory agencies such as the FDA and by NIH. This analysis includes preclinical evaluation of the adjuvant alone plus evaluation of the antigen-adjuvant combination to assess risks due to the combination that might not be noted in the adjuvant alone (116). It is recommended that the species used for toxicology testing of an adjuvant be sensitive to the biological effects of the adjuvant. If possible, the animals are dosed with equal or more than the absolute dose planned for human use, with the number of doses designed to be one more than that intended for human use and at intervals appropriate to closely simulate the clinical setting. Safety related assessments include and are not limited to pre- and post-vaccination serum chemistry (including liver/renal function tests and creatine kinase) and hematology, injection site observations, injection site histopathology, limb use impairment and complete necropsy. Other tests specific to the mechanism of a given adjuvant are also performed. For example, enterotoxin-type adjuvants are evaluated for uptake into the olfactory nervous system to rule out this possibility due to a high proportion of GM1 (toxin receptor) in this tissue.

8.2. Clinical experience with experimental vaccine adjuvants

Among the most extensively evaluated adjuvants are MPL, MF59, QS-21, and AS02, all of which have been tested in multiple vaccines and clinical trials in hundreds to thousands of individuals. Table 5 outlines some of the trials in which these adjuvants have been evaluated. This is not limited to these trials. MPL has been evaluated in vaccines for HSV (in combination with alum (117)), a melanoma vaccine ("Melacine" (118)), and others. MF59 has been evaluated in vaccines for influenza (119), HSV type 2 (120), hepatitis B (121) and HIV (122). QS-21 has been evaluated in various cancer immunotherapies (86), in HIV vaccines (123), malaria vaccines (124), and vaccines for other indications. AS02 has been tested extensively in malaria vaccines including Phase II trials (125), in HIV vaccines (126), and therapeutic hepatitis B vaccines (127).

8.3. Licensed vaccines containing novel adjuvants

Until recently, the only adjuvant used in licensed human vaccines was aluminum hydroxide or related mineral salt adjuvants. A few vaccines with novel immunostimulants have now been licensed. MF59 – adjuvanted influenza vaccines are licensed in Europe based upon a clinical database of 12,000 subjects (128). Virosomal influenza and hepatitis vaccines are also licensed in some European countries (129). We can expect that additional vaccines with novel adjuvants will be licensed in the near future as many adjuvanted vaccines are now in late-stage clinical trials.

9. CONCLUSIONS

Current vaccine adjuvants cover a broad and diverse class of compounds, including natural products, synthetic, and semisynthetic compounds. Many of these were discovered through traditional trial and error processes. Adjuvant discovery and evaluation will continue to play a significant role in vaccines of the future. Identification and understanding of PAMP-PRR interactions will aid in the development of new adjuvants. With this improved understanding and improved adjuvant screening methods, we move away from trial and error discovery of adjuvants and closer to rational design of new adjuvants.

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