Evolution of Adjuvants Across the Centuries

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Adjuvants are substances that are added to vaccine antigens to enhance and modulate the immunogenicity of the antigen. The first adjuvants developed focused on increasing antibody responses, and this has often been sufficient for the vaccines considered. During the last 2 decades, however, it has been realized that simply increasing antibody responses is not always sufficient for candidate vaccines to be effective. It has been observed that adjuvants can be used very effectively to:

* provide a strong priming response in naïve populations, effectively reducing the number of doses required to induce protection.
* increase the duration of the immune response.
* enhance specific arms of the immune response such as cell- mediated immunity (CMI), a critical target for many of the remaining infectious diseases for which we do not have vaccines.
* increase the breadth of the immune response to variable antigens, enabling broader cross-protection.
* enhance the immune response in poorly responsive populations, such as elderly and immunosuppressed populations.
* allow for dose sparing of antigens where antigen supply is limited.

Generally speaking, adjuvants are useful for antigens such as inactivated, subunit, and recombinant proteins, which can lose, during the purification process, some of the immunologi- cal information present in the pathogen that are needed to trigger an immune response. They have not yet been required for live attenuated vaccines, which carry the necessary immune- stimulating signals themselves. As discussed later, however, some preliminary research suggests that adjuvants can have an effect on live vaccines as well.

# CHANCE AND NECESSITY: THE DISCOVERY OF ADJUVANTS

The use of adjuvants has been known for more than a century, and it is only recently that their mechanism of action was elucidated, in part owing to the progress in microbiology and immunology. The first recorded observation of immune potentiation by “adjuvants” is probably that of Coley, who in 1893 observed that administration of killed bacteria (Coley toxins) could in some cases cure certain forms of cancer. It was only in the 1990s that it was determined that this effect was the result of immune stimulation mediated by bacterial DNA. From there on, the specific oligonucleotide sequences that could stimulate the immune response and enhance it to a coadministered antigen were discovered.

It took another 2 decades to recognize the usefulness of adjuvants to enhance humoral immunity. In 1925, Ramon1 observed that administering diphtheria toxoid to horses with a variety of substances, including starch, plant extracts, or fish oils, substantially enhanced the antibody response to the toxoid. A year later, Glenny2 observed a similar effect with aluminum potassium sulfate, or alum. Alum was used there- after as an adjuvant for numerous human vaccines, and to this

day, other aluminum salts, in the form of aluminum oxyhy- droxide or hydroxyphosphate, are the most widely used adju- vants in human vaccines. The starch and fish oils shown by Ramon to act as adjuvants have, in recent decades, been tested in vaccines in the form of inulin and squalene, respectively.

During the 80 years following the first use of aluminum salts as adjuvants, a wide variety of substances were tested as adjuvants, but many of them failed to be accepted for human use. In the 1940s, Jules Freund developed a water-in-oil emul- sion, the Freund adjuvant, in which the vaccine antigen is emulsified as water droplets in a continuous mineral oil phase, containing killed mycobacterium (Freund complete adjuvant) or not (Freund incomplete adjuvant). The latter was briefly used for a commercial influenza vaccine in the United Kingdom in the 1960s, but was soon withdrawn owing to unacceptable reactogenicity. This, however, led to the develop- ment of oil-in-water emulsions, in which oil droplets are present in a continuous aqueous phase.

The first oil-in-water emulsions were based on a nonme- tabolizable oil (squalane) and replaced later with metaboliz- able oils (squalene), as opposed to mineral oils as in the original Freund adjuvant. However, a water-in-oil emulsion similar in structure to the Freund adjuvant has been introduced in a cancer vaccine, using mineral oil with a higher degree of purity that allows for use in human vaccine candidates.

In the 1970s liposomes and virosomes that adsorb or encapsulate antigen were developed. Liposomes consist of lipid layers that form nanospheres or microspheres and can encapsulate or integrate antigens into their membranes. Several licensed vaccines contain virosomes, which are recon- stituted empty envelopes of influenza viruses similar in struc- ture to liposomes.

# A TURNING POINT: BETTER UNDERSTANDING OF IMMUNOLOGY AND ITS IMPACT ON THE DEVELOPMENT OF ADJUVANTS

For most of the 20th century, adjuvant discovery and develop- ment was based on observations and experimentation with no clear immunological knowledge of the mechanism behind the adjuvant effect. This, however, dramatically changed in 1996 with the discovery of the Toll-like receptors (TLR) family in *Drosophila* and their relation to fungal resistance by Lemaitre and colleagues.3 One year later, in 1997, Janeway4 identified the link between human TLR4 and its key role in initiating an adaptive immune response, the first necessary step to a long-lasting immune response. The discovery by Poltorak and colleagues5 that TLR4 functioned as a lipopoly- saccharide (LPS)-sensing receptor and, hence, the use of LPSs or their derivatives as adjuvants, brought the final piece to the understanding of the mechanism of actions of TLR agonist molecules.6

In the early 1980s, Edgar Ribi7 established that it was pos-

sible to produce a molecule that retained the immune poten- tiation activity of LPS without the associated toxicity. Approximately 30 years later, in 2009, the molecule mono- phosphoryl lipid (MPL) A was the first new adjuvant in a vaccine (Cervarix vaccine against human papillomavirus

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[HPV]) approved for use by the U.S. Food and Drug Administration.

It is now well understood that the immune system uses pathogen-associated molecular patterns (PAMPs) to activate pathogen-recognition receptors such as TLR, and also a host of other more recently discovered receptors: retinoic-acid– inducible gene-based-I–like receptors (RLRs),8 and cytosolic nucleotide oligomerization domain (NOD)-like receptors (NLRs).9,10 These receptors bind various pathogen ligands (ranging from, for example, bacterial cell wall and cell mem- brane components to bacterial or viral nucleotides, to fungal lipids) to trigger different types of immune responses and, if combined with an antigen, can initiate and enhance specific arms of the immune responses to that antigen. For the purpose of brevity, readers are directed to other sources for a detailed description of how pathogen components stimulate various cytokine pathways and how they direct different arms of the immune response11; [Fig. 6.1](#_bookmark0) shows this information schematically. As shown in [Fig. 6.1](#_bookmark0), different TLRs, located on the plasma membrane or intracellularly, respond to

different pathogen-derived signals to induce proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, or type 1 interferon, leading to a predominantly T-helper cell 1 (Th1) response. However, certain TLR2 agonists have been reported to activate Th2 responses.12

[Table 6.1](#_bookmark1) lists the various agonists that activate the TLRs, as well as the adaptor molecules, also known as TLR agonists, and examples of adjuvants, which function through these receptors.

Based on this understanding, it is possible to recognize today how most of the adjuvants function. This knowledge should allow rapid screening of compound libraries for mol- ecules that bind these receptors and that may have adjuvant activity leading to the rational design of new adjuvants aimed at stimulating specific arms of the immune response. The level of knowledge associated with the mechanism of action of those specific molecules and, as a consequence, the pattern of cytokines induced should also allow an assessment of the impact of an adjuvant on the safety of a vaccine. However, this has not yet led to the discovery of new molecules with defined

TRAM TRIF MyD88 TIRAP

MyD88

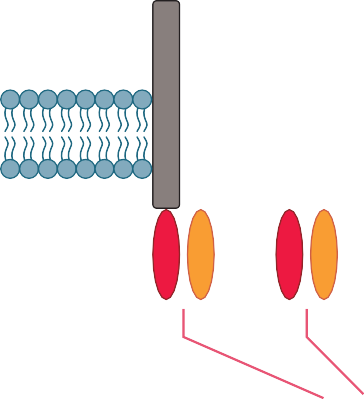
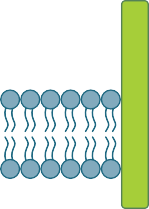
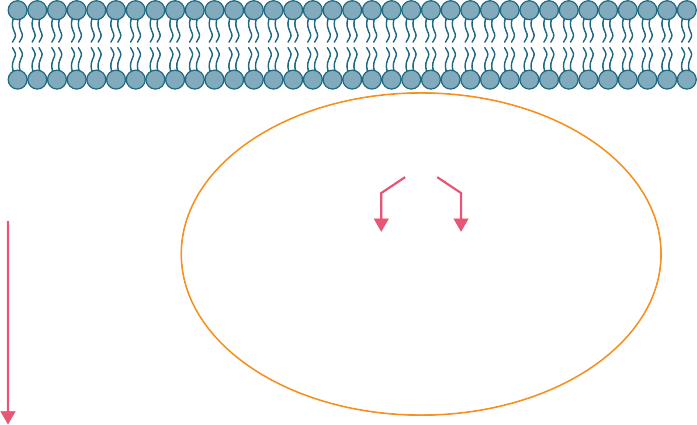
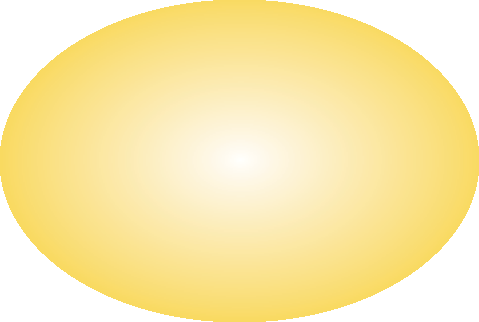
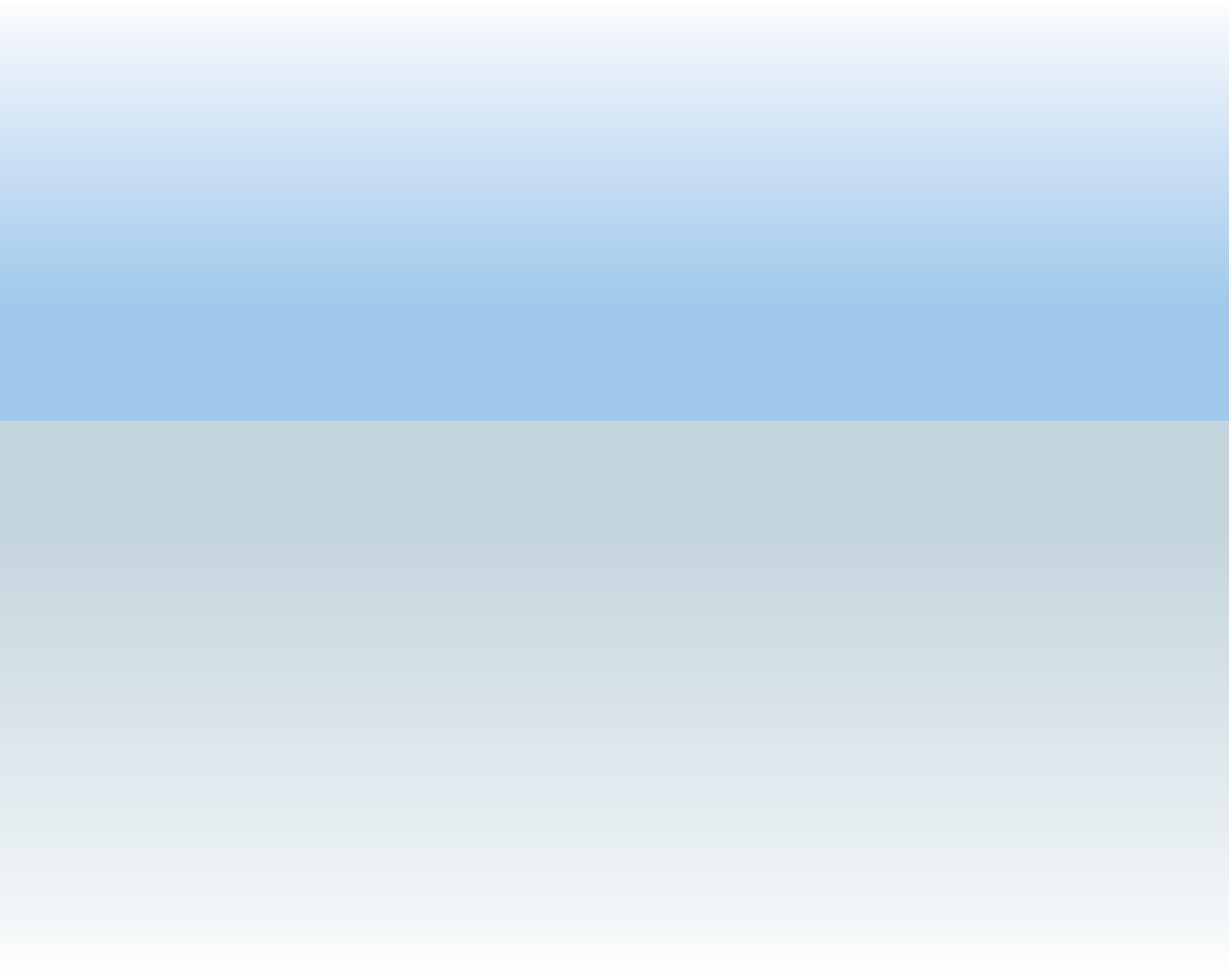
MyD88

TIRAP

MyD88

TIRAP

**Figure 6.1.** Activation of the innate immune system via interaction of bacterial or viral components with specific Toll-like receptors (TLRs), mes- saging through the myeloid differentiation 88 (MyD88) or TRIF pathway, and subsequent secretion of interleukin (IL)-10, tumor necrosis factor (TNF)-α and IL-6, or type 1 interferon. dsDNA, double-stranded DNA; IRAK, interleukin receptor–associated kinase; IRF, interferon regulatory factor; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; ssRNA, single-stranded RNA; Th, T-helper cell; TRAF, TNF receptor–associated factor; Treg, T-regulatory cell; TRIF, TIR domain–containing adapter-inducing interferon-β.



8

IRF-7

9

IRAK-TRAF

Endosome

MAPK

NF-B

NF-B

IL-10

TH2/Treg

TNF-, IL-6

Th1

Interferon Th1

7

TLR 3

DNA

dsDNA ssRNA

MD2

TLR4

TLR2/TLR1 TLR2/TLR6 TLR5

LPS

Lipoteichoic Lipopeptides acid Flagellin

Virus

Bacteria

MyD88

MyD88

MyD88

TRIF

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| **TABLE 6.1** Agonists That Activate the TLRs, Adaptor Molecules, and Examples of Adjuvants | | | | |
| **TLR** | **Ligand** | **Ligand Location** | **Adaptor** | **Adjuvant** |
| 1 | Tripalmitoyl-cysteine lipopeptides | Bacterial membranes | MyD88 MAL |  |
| 2 | Lipopeptides, β-glucan, glycolipids | Bacterial membranes | MyD88 MAL |  |
| 3 | Double-stranded RNA | Viral RNA | TRIF | Poly(I:C); poly(A:U) |
| 4/MD2 | Lipopolysaccharide | Bacterial membranes | MyD88 MAL/TRIF | MPL, GLA, E6020, RC529 |
| 5 | Flagellin | Bacterial outer surface | MyD88 | VaxInnate |
| 6 | Dipalmitoyl-cysteine lipopeptides | Bacterial membranes | MyD88 MAL |  |
| 7 | Single-stranded RNA |  | MyD88 | Imidazoquinolines: imiquimod, loxoribine |
| 8 | Single-stranded RNA |  | MyD88 | R848 |
| 9 | Bacterial DNA, unmethylated CpG DNA sequences, poly(dI:dC) | Bacteria | MyD88 | CpG; IC-31 CpG 1018 |
| CpG, cytosine phosphate guanine; GLA, glucopyranosyl lipid adjuvant; MAL, MyD88 adaptor-like; MPL, monophosphoryl lipid A; MyD88, myeloid differentiation 88; TLR, Toll-like receptor; TRIF, TIR domain-containing adaptor-inducing IFN-β. | | | | |

activities, but in certain cases has enabled a better understand- ing of the mode of action of specific adjuvants and helped to support their safety profile within a given vaccine.13 A clear understanding of the pathogenesis of immune-mediated dis- orders and their triggers is required, however, to ascertain the potential impact of the adjuvant. For several adjuvants the exact mechanism of action remains elusive (such as the sapo- nins, which are described later) or may present multiple modes of action (such as aluminum salts).

This is only the first step in defining the value of a molecule as an adjuvant. Further evaluation of the compound in vivo and its safety profile will define its real potential as an adju- vant for vaccines.14,15

# DEFINING ADJUVANTS: CLASSIFICATION AND EVALUATION

This section is limited to a review of the types of adjuvants that are incorporated in licensed vaccine formulations or for which there is extensive clinical experience.

# A Categorization of Adjuvants Based on Mechanism of Action

The majority of adjuvant reviews during recent decades have tried to classify adjuvants according to their mechanism of action and typically classified adjuvants as *vehicles* or *immuno- stimulants*. Immunostimulants are substances that act directly on the immune system, such as TLR ligands. Vehicles are thought to act primarily by presenting antigens to the immune system. In this group are various aluminum salts, emulsions, immunostimulatory immune complexes (ISCOMs), and bio- degradable microparticles. It is now known that most of the vehicles act directly on the immune system, and antigen pre- sentation may be only a minor component of the adjuvant activity (e.g., see the later discussions of modes of action of aluminum salts [“[Aluminum Salt Adjuvants](#_bookmark2)”] and oil-in-water emulsions [“[Oil-in-Water Emulsions](#_bookmark9)”]). This classification seems outdated, and it may be preferable to classify adjuvants according to their receptor or, when the receptor is unknown, by their physical or chemical nature.

Currently 10 adjuvants are approved for use in vaccines

(three aluminum salts with different counter-ions, four oil-in- water emulsions, aluminium/MPL combination, virosomes,

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and polyoxidonium). [Table 6.2](#_bookmark3) lists the approved vaccines containing these adjuvants. Numerous other adjuvants are in vaccines that are under development. Noteworthy, AS01, the combination of liposome, MPL and QS21, will most likely be the next new adjuvant present in a licensed vaccine, in this case the malaria RTS,S vaccine. These and their classifications by receptor or physicochemical nature are presented in [Table](#_bookmark8)

[6.3](#_bookmark8) and discussed in the following sections. Many more adju- vants are in preclinical development; however, they are too numerous to discuss in this chapter.16

## Aluminum Salt Adjuvants

Aluminum-containing adjuvants have historically served as immunostimulants in vaccines and continue to be the most widely used adjuvants. Several aluminum compounds are used and are known as aluminum hydroxide, aluminum phos- phate, and alum. All three of these commonly used names are scientific misnomers. Although this family of adjuvants has been used the longest, it is only recently that we have begun to understand their mechanism of action and the complexity of formulating them with antigens.

The following sections summarize the structure and prop- erties of different aluminum salts, the mechanisms by which they stimulate the immune response, and the effect of freezing on aluminum-adjuvanted vaccines.

**Structure and Properties.** Aluminum hydroxide adjuvant is not Al(OH)3, but rather crystalline aluminum oxyhydroxide (AlOOH).17 This difference is important because crystalline aluminum hydroxide has a low surface area (approximately 20 to 50 m2/g) and as such is a poor adsorbent. Crystalline aluminum oxyhydroxide has a surface area of approximately 500 m2/g,18 which makes it an excellent adsorbent. This high surface area is a result of its morphology. The primary particles are fibers having dimensions of approximately 5 × 2 × 200 nm.

Aluminum oxyhydroxide is a stoichiometric compound. The surface is composed of Al-OH and Al-O-Al groups. The Al-OH surface groups can accept a proton, resulting in a posi- tive surface charge, or donate a proton, resulting in a negative surface charge. As shown in [Fig. 6.2](#_bookmark7), the isoelectric point (IEP) of Al-OH is 11.4. Thus, aluminum oxyhydroxide exhibits a positive surface charge at pH 7.4, which is the pH of interstitial fluid.

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| **TABLE 6.2** Types of Licensed Vaccines Containing Adjuvants | | |
| **Vaccine** | **Trade Namea** | **Adjuvant** |
| Diphtheria and tetanus vaccine (DT) | Diphtheria and Tetanus Toxoid Adsorbed USP (1) | Aluminum potassium phosphate |
| DT acellular pertussis (DTaP) | Tripedia (1) | Aluminum potassium phosphate |
| *Haemophilus influenzae* type b (Hib) | Liquid PedvaxHIB (2) | Aluminum hydroxyphosphate sulfate |
| DTaP + Hib | TriHIBit (1) | Aluminum potassium phosphate |
| Hepatitis B | Recombivax HB (2) | Aluminum hydroxyphosphate sulfate |
| Hepatitis B | Engerix-B (3) | Aluminum hydroxide |
| Hepatitis B + Hib | Comvax (2) | Aluminum hydroxyphosphate sulfate |
| Hepatitis A | Havrix (3) | Aluminum hydroxide |
| Hepatitis A | Epaxal (6) | Virosomes |
| Hepatitis A + hepatitis B | Twinrix (3) | Aluminum hydroxide/phosphate |
| Pneumococcal conjugate vaccine | Prevnar (4)  Synflorix (3) | Aluminum phosphate |
| Influenza vaccine | FLUAD (4)b | MF59 |
| Influenza vaccine | Inflexal V (6)b | Virosomes |
| Pandemic influenza vaccine | Pandemrix | AS03 |
| Pandemic influenza vaccine | Focetria | MF59 |
| Pandemic influenza vaccine | Humenza | AF03 |
| Human papillomavirus (HPV) | Gardasil (2) | Aluminum hydroxyphosphate sulfate |
| HPV | Cervarix (3) | Aluminum hydroxide + MPL |
| Hepatitis B | Fendrix (3)b | AS04 (MPL + aluminum phosphate) |
| Hepatitis B | SUPERVAX (7)b,c | RC529 |
| MPL, monophosphoryl lipid A.  aManufacturers are as follows: 1, Sanofi Pasteur; 2, Merck; 3, GlaxoSmithKline; 4, Wyeth now Pfizer; 5, Novartis; 6, Crucell; 7, Dynavax Europe.  bLicensed in Europe.  cLicensed in Argentina. | | |

**Figure 6.2.** Isoelectric points of aluminum hydroxide adjuvant *(right)* and aluminum phosphate adjuvant *(left)*. *(From Rinella JVJ, White JL, Hem SL. Effect of pH on the elution of model antigens from aluminum-containing adjuvants.* J Colloid Interface Sci. *1998;205: 161–165.)*



40

20

0

–20

–40

2

4

6

8

10

12

pH

Zeta potential (mV)

Aluminum phosphate adjuvant is a chemically amorphous aluminum hydroxyphosphate in which some of the hydroxyl groups of aluminum hydroxide are replaced by phosphate groups. The disordered, amorphous state is responsible for the high surface area and high adsorptive capacity.

The surface of aluminum phosphate adjuvant is composed of Al-OH and Al-OPO3 groups. The IEP varies from 9.4 to 4.5 depending on the degree of phosphate substitution.19 Com- mercial aluminum phosphate adjuvants have IEP values in the

4.5 to 5.5 range. In contrast with aluminum oxyhydroxide, commercial aluminum phosphate adjuvants are negatively charged at pH 7.4 (see [Fig. 6.2](#_bookmark7)).

Alum, which is water-soluble, is chemically aluminum potassium sulfate, AlK(SO4)2. The earliest vaccines containing aluminum adjuvants were prepared by in situ precipitation. A solution of alum was mixed with a solution of the antigen dissolved in a phosphate buffer. It is common practice to refer to the adjuvant produced by in situ precipitation as alum. The precipitate is amorphous aluminum hydroxyphosphate and has similar composition and properties as aluminum phos- phate adjuvant.17,20

The techniques that can be used to characterize aluminum- containing adjuvants have been reviewed by White and Hem.21

**Effect of Freezing.** Vaccines containing aluminum hydroxide adjuvant or aluminum phosphate adjuvant should not be allowed to freeze and should not be used if suspected of having been exposed to freezing temperatures.22 Freezing may affect the aluminum-containing adjuvant and the adsorbed antigen. Coagulates, which cannot be redispersed by shaking, form when aluminum-containing adjuvants are frozen. Ther- mostability of vaccines is of increasing importance, and as demonstrated for aluminium salts does not only concern excursion at high temperature, but also at low temperature, such as freezing.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **TABLE 6.3** Nonexhaustive List of Adjuvants in Vaccines Under Development or Approved | | | | | | | |
| **Class (By MOA)** | **Component** | **Adjuvant Name and Other Compounds** | **Manufacturer** | **Phase I** | **Phase II** | **Phase III** | **Licensed** |
| Hiltonol (polylysine) Oncovir Cancer | | | | | | | |
| TLR4 | MPL MPL MPL MPL MPL RC529 GLA | AS04 (alum)  AS02 (emulsion, QS21) AS01 (liposome, QS21)  AS15 (liposomes QS21, CpG) (tyrosine)  RC529 (alum)  SE-GLA (emulsion) Stimuvax  E6020 | GSK GSK GSK GSK ALT GSK IDRI  Biomira/Oncothyreon Esai | Influenza | Cancer | Malaria, zoster Cancer  Allergy  Cancer | HPV, HBV  HBV |
| TLR5 | Flagellin | Fusion to influenza hemagglutinin | VaxInnate | Influenza |  |  |  |
| TLR7 | Imiquimod Imiquimod | Topical  Combined with ISA51 |  | Cancer | Cancer |  |  |
| TLR9 | CpG  dI:dC | 1018 ISS  CpG 7909  CpG 7909 + alum  AS15 (liposomes, MPL, QS21) IC31 (cationic peptide) | Dynavax Coley/Pfizer NIAID  GSK  Intercell | HBV  Malaria  Influenza, TB | Allergy Cancer  Cancer | HBV |  |
| Saponins | QS21 | AS01 (liposome, MPL) | GSK |  |  | Malaria |  |
|  | QS21 | AS15 (MPL, CpG) | GSK |  |  | Cancer |
|  | QS21 | QS21 | Universities | HIV, influenza | Cancer |  |
|  | Quil fractions | ISCOM (cholesterol) | CSL |  |  |  |
|  |  | Iscomatrix (cholesterol) | CSL | HPV, influenza | Cancer |  |
|  | GPI-0100 |  |  | Cancer |  |  |
| Oil-in-water emulsion | Squalene Squalene  Tocopherol Squalane | MF59 AF03 SE  AS03 (squalene)  CoVaccine (acyl sucrose sulfate) | Novartis Sanofi Pasteur IDRI  GSK  Protherics Nobilon | HIV  Influenza Influenza  Angiotensin Influenza | HBV, CMV |  | Seasonal influenza, pandemic influenza  Pandemic influenza |
| Water-in-oil emulsion | Squalene | ISA 720  ISA 51 | Seppic Seppic | Malaria |  | Malaria, cancer Cancer |  |
| Polysaccharides | Inulin | Advax (alum) | Vaxine | HBV, influenza |  |  |  |
| Cationic liposomes | DDA | CAF (TDM) JVRS-100 (DNA) | SSI  Juvaris | TB  Influenza |  |  |  |
| Virosomes |  |  | Crucell Pevione | Malaria |  |  | HAV, influenza |
| Polyelectrolytes |  | Polyoxidonium | Microgen |  |  |  | Influenza |
| Classified according to adjuvant receptor or adjuvant physicochemical nature (alum-derived adjuvants excluded for clarity).  ALT, alanine aminotransferase; CMV, cytomegalovirus; CpG, cytosine phosphate guanine; DDA, dimethyldioctadecylammonium; GLA, glucopyranosyl lipid adjuvant; GSK, GlaxoSmithKline; HAV, hepatitis A virus; HBV, hepatitis B virus; HPV, human papillomavirus; IDRI, Infectious Disease Research Institute; ISCOM, immunostimulatory immune complex; MOA, mechanism of action;  MPL, monophosphoryl lipid A; NIAID, National Institute of Allergy and Infectious Diseases; SE, stable emulsion; TB, tuberculosis; TLR, Toll-like receptor. | | | | | | | |

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**Adsorption Mechanisms.** The major mechanisms responsi- ble for the adsorption of antigens are electrostatic attraction, hydrophobic forces, and ligand exchange. Electrostatic attrac- tion is probably the most frequently used adsorption mechanism.

Electrostatic attraction can be optimized by determining the IEP of the antigen and then selecting an adjuvant that will have the opposite surface charge at the desired pH. For example, at pH 7.4, aluminum hydroxide adjuvant (IEP = 11.4) adsorbs albumin (IEP = 4.8) but does not adsorb lyso- zyme (IEP = 11.0). In contrast, aluminum phosphate adjuvant (IEP = 4.0) adsorbs lysozyme but not albumin at pH 7.4.23

Care must be taken in selecting a buffer for an aluminum hydroxide adjuvant–containing vaccine. Electrostatic attrac- tion for an acidic antigen may be reduced or reversed if a phosphate buffer is used. Acetate and tromethamine (TRIS) are examples of buffers that do not alter the IEP of aluminum hydroxide adjuvant.17

Aluminum hydroxide adjuvant can also be pretreated to lower the IEP to optimize electrostatic adsorption of basic antigens.24

Hydrophobic forces can also contribute to the adsorption of antigens by aluminum-containing adjuvants. The contribu- tion of hydrophobic attractive forces can be determined by observing the effect of ethylene glycol on adsorption.25 Ethylene glycol stabilizes the hydration layer of proteins, which renders hydrophobic interactions thermodynamically unfavorable.

**Mechanisms of Action of Aluminum Salt Adjuvants.** There is still no consensus regarding the mechanisms by which aluminum-containing adjuvants potentiate the immune response. Several mechanisms are frequently cited to explain how aluminum-containing adjuvants increase antibody pro- duction. The depot mechanism was initially thought of as the dominant one; later the promotion of uptake of antigens by antigen-presenting cells (APCs)26 and, more recently, a direct immune-stimulating mechanism were proposed.

* The depot mechanism postulates that the aluminum- containing adjuvant and the adsorbed antigen remain at the site of injection. The antigen is released slowly to stimulate the production of antibodies. This hypothesis is supported by the observation with some antigens that stronger binding to the aluminum salt crystals can result in higher immune responses. This hypothesis is, however, inconsistent with the observation that alum injection sites can be excised shortly after injection with no impact on immunogenicity.27
* It has also been proposed that adsorption of antigen to aluminum-containing adjuvants converts the soluble antigen to a particulate form. APCs take up particulate matter more efficiently by phagocytosis. Thus, antigen, which remains adsorbed, is taken into macrophages and dendritic cells. A dendritic cell culture study28 revealed that antigens that elute from the aluminum-containing adju- vants are internalized by macropinocytosis, while those that remain adsorbed are internalized by phagocytosis. Antigen internalization by dendritic cells was enhanced when the antigen remained adsorbed to the aluminum-containing adjuvant following administration and when the aggregate size of the adjuvant was smaller than dendritic cells.
* Several groups have identified a role for a direct stimulation of the immune system through innate immune receptors and identified activation of the Natch domain, leucine-rich repeat, and PYD-containing protein (NALP)-3 inflamma- some pathway by alum as the mechanism of action.29–31 The precise mechanism by which alum stimulated NALP3 remained unknown. This has been refined and alum crystals

have been shown to interact directly with membrane lipids on the surface of dendritic cells. The resulting lipid sorting triggers signaling cascades, independent of the inflamma- some, that promote CD4+ T-cell activation.32

It is likely that all three proposed mechanisms contribute to the immunostimulation produced by aluminum-containing adjuvants.

**Safety of Aluminum-Containing Vaccines.** Aluminum salts as adjuvants have the longest and largest safety track record of all adjuvanted vaccines, with more than 3 billion vaccine doses used during the past 80 years with a positive risk-to- benefit ratio. Focal histologic lesions were observed in patients with diffuse muscular symptoms that included persistent myalgias, arthralgias, and persistent fatigue. In the approxi- mately 130 cases studied, these lesions were identified as mac- rophagic myofasciitis (MMF).33 Intracytoplasmic inclusions in the infiltrating macrophages have been identified as contain- ing aluminum by electron microscopy, microanalysis, and atomic adsorption spectroscopy. The presence of aluminum in the deltoid muscle biopsies suggested to Gherardi and col- leagues that the source of the aluminum was aluminum hydroxide adjuvant.34 However, no relationship between the presence of aluminum and MMF and the clinical symptoms has been established. The Vaccine Safety Advisory Committee of the World Health Organization (WHO) reviewed MMF at a meeting in 1999. The committee found that there was no basis for recommending a change in vaccination practices involving vaccine selection, schedule, delivery practices, or information involving aluminum-containing vaccines. The committee recommended that “research studies be under- taken to evaluate the clinical, epidemiological, and basic science aspects of MMF.”35 The U.S. Food and Drug Adminis- tration, while recognizing the desirability of new adjuvants, confirmed its support of aluminum salts in vaccines.36 Research studies undertaken to assess the neurotoxicity of aluminum when it is administered intramuscularly or in a vaccine, showed a difference between the control group and the aluminum-based vaccine tested.37 A repeat of the experiment, however, did not confirm any differences between the control group and the two vaccines containing aluminum. To date, even though it is established that aluminium salt can be recov- ered at the injection site months or years after intramuscular injections, no link between the presence of aluminium salt and the MMF syndrome has been clearly established.

## Water-in-Oil Emulsions

Water-in-oil emulsions, of which the Freund adjuvant is the best-known example, were included in a commercial influenza vaccine in the United Kingdom in the 1960s. The vaccine was later withdrawn owing to occasional abscesses observed at the site of injection. Initial large-scale clinical studies on 18,000 military recruits conducted in 195338,39 resulted in some nodules at the injection sites, which were attributed to impuri- ties (short-chain fatty acids) in the Arlacel-A surfactant. However, when this surfactant was purified, the incidence of cysts was reduced. A 10-year follow-up on these volunteers40 showed that cyst-like reactions had required hospitalization in 0.1% to 0.6% of the volunteers, but otherwise there were no adverse effects of the vaccine. A subsequent 35-year follow-up41 demonstrated that not only were there no adverse correlations with different diagnoses, including autoimmune diseases, but also, for some of the disease categories, there was decreased mortality. In contrast with these data from a large clinical trial, studies in rodents in 1972 demonstrated that when male Swiss mice were injected with mineral oil–based

emulsions, the mice developed tumors,42 and the unaccept- ability of mineral oil–based emulsions for human use was concluded.

As a result, water-in-oil emulsions based on metabolizable oil were developed using squalene instead of mineral oil. The best-known examples of these are the Montanide adjuvants such as ISA 720 produced by the company Seppic. ISA 720 water-in-oil emulsion has been widely tested in more than 70 clinical trials in which it was often shown to induce immune responses rarely surpassed by other adjuvants. However, as with mineral oil–based emulsions, cysts or sterile abscesses at the site of injection are not infrequent and tend to increase in frequency on boosting.43 In addition, instability of the antigen in contact with the emulsion was observed.44 Finally, the dif- ficulty in performing reproducible formulation at the time of administration led to a preformulation that may be incompat- ible with antigen stability. These challenges suggest that for prophylactic vaccines, researchers should, when possible, avoid using water-in-oil emulsions. For therapeutic vaccines, however, the risk of cysts and the challenges of formulation may be less significant. Such a vaccine (CIMAvax), which con- tains a mineral oil–based water-in oil-emulsion, Montanide ISA 51, has been licensed in Cuba for non–small cell lung cancer.45

## Oil-in-Water Emulsions

Oil-in-water emulsion adjuvants were initially developed as an alternative to water-in-oil emulsions; the lower viscosity makes them easier to inject. The first emulsion of this class to be developed for human use was the SAF adjuvant made by Syntex Corporation. This emulsion was based on nonbiode- gradable squalane, the catalytically hydrogenated form of squalene, and was designed as a vehicle to carry a synthetic immunostimulant, threonyl muramyl dipeptide (MDP).46 The SAF adjuvant, while displaying strong adjuvant activity, was too reactogenic for use, partially because of MDP. However, because it was less effective without the immunostimulant, the emulsion was abandoned. Later, Chiron Corporation devel- oped a range of oil-in-water emulsions by replacing squalane with squalene as another vehicle for muramyl derivatives. One of these emulsions (MF59) demonstrated some adjuvant properties and was further evaluated. MF59 and the majority of the later-developed oil-in-water emulsions used squalene, a natural, metabolizable product found in all plant and animal cells where it is a precursor of cholesterol. The com- mercial source is generally from shark liver, where it is abun- dant; alternative sources such as phytosqualene are being explored.47 However, to date, only squalene from shark origin allows for a product with a purity level acceptable for human use.

Despite extensive clinical studies with a wide range of anti-

gens, MF59 was approved only in one vaccine, FLUAD, an influenza vaccine for older adults, and licensed in several European countries from 1997 onward. While there was benefit of the adjuvanted vaccine in terms of antibody response to the influenza hemagglutinin in the target population,48 the really significant benefit of MF59 and other oil-in-water emul- sions became clear during investigations on pandemic influ- enza vaccines. The emergence of avian H5N1 influenza with occasional human-to-human transmission and the fear that this could become a pandemic led to intensive research in academic and pharmaceutical environments for ways to immunize a largely immunologically naïve population in the context of limited antigen supplies. This was especially critical when it was shown that for an H5N1 pandemic strain, sixfold more antigen was required to induce an immune response to a level equivalent to the seasonal influenza vaccine (90 µg

compared with 15 µg).49 It was shown that MF59 enabled immunization with significantly reduced doses of antigen, down to 7.5 µg, nearly a 12-fold dose reduction.50

In parallel to the development of MF59, several other oil-in-water emulsions were developed. For example an oil-in- water emulsion containing α-tocopherol as the immunostim- ulating compound was formulated by GlaxoSmithKline. This emulsion was tested earlier as part of the initial development of a malaria vaccine, alone (AS03) or in combination with the immunostimulants MPL and QS21 (described later).51 AS03 demonstrated potent dose-sparing potential for pan- demic influenza antigens, allowing for dose sparing down to

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3.75 µg.52

In response to the need for dose sparing and the demon- strated potential of oil-in-water emulsions, Sanofi Pasteur developed AF03. This adjuvant is also squalene based; however, unlike the other emulsions, which are made by microfluidiza- tion of the components, the emulsification of AF03 is achieved without mechanical energy and uses a temperature-induced self-emulsification process (PCT application WO2007080308). With the outbreak of the H1N1 influenza pandemic, Euro- pean regulatory authorities approved three oil-in-water emul- sions containing pandemic influenza vaccines, with MF59,

AS03, and AF03 as adjuvants.

Other emulsions are under development, such as SE (stable emulsion), a squalene-based emulsion, originally developed by researchers at Corixa as a vehicle for MPL and synthetic TLR4 agonists.53 This emulsion differs from the others in that the emulsifier is a natural phospholipid rather than a surfac- tant such as Tween-80. SE has been tested in clinical trials in combination with MPL in the context of a *Leishmania* vaccine,54 as well as in combination with the TLR-4 agonist GLA (gluco- pyranosyl lipid adjuvant) in a schistosomiasis vaccine,55 and in an influenza vaccine.56 CoVaccine is an experimental adju- vant comprising sucrose fatty acid sulfate ester, combined with squalane, in the form of an oil-in-water emulsion. This adju- vant has been reported to allow for dose sparing in the context of influenza vaccines.57 A single immunization with CoVaccine HT-adjuvanted H5N1 influenza virus vaccine induces protec- tive cellular and humoral immune responses in ferrets and is undergoing clinical evaluation.

[Table 6.4](#_bookmark10) gives an overview of oil-in-water emulsions used

as adjuvants in licensed and investigational vaccines.

**Adjuvant Effect of Oil-in-Water Emulsions on Naïve Versus Primed Persons.** Oil-in-water emulsion adjuvants are highly effective at enhancing immunogenicity and allowing for dose reduction in pandemic influenza vaccines when the vaccinees are naïve. In contrast, the adjuvant effect for seasonal vaccines in healthy adults is quite poor.58 This suggests that these adju- vants are excellent for priming but do not boost efficiently preexisting immune responses. The benefit for priming is also evident in the studies on the use of MF59-adjuvanted seasonal vaccine in infants, who usually respond poorly to a single administration of seasonal vaccine. These studies show a strong effect of MF59 on immunogenicity59 and on efficacy of seasonal influenza vaccines, with the effect being strongest in the youngest ages.60 The adjuvanted vaccine demonstrated 89% efficacy against vaccine-matched strains during two influ- enza seasons compared with 45% for the nonadjuvanted sea- sonal influenza vaccines group. On the basis of these data, Canada approved in early 2015 the use of MF59-adjuvanted seasonal influenza vaccine (FLUAD Pediatric) for the pediatric population (6 months to 2 years of age).

The situation is slightly different in the elderly population.

Older adults have, in general, been primed to seasonal influ- enza; however, immunological senescence results in a decreased ability to induce sufficient antibody responses to

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| **TABLE 6.4** Composition of Various Oil-in-Water Emulsions | | |
| **Name** | **Components** | **Regulatory Status** |
| SAF | Squalane; block copolymer; MDP | Abandoned |
| MF59 | Squalene; Tween-80; Span 85 | Approved for seasonal influenza for elderly people; approved for pandemic influenza (by the EMA); clinical benefit demonstrated for seasonal influenza in infants |
| AS03 | Tocopherol; squalene; Tween-80 | Approved for pandemic influenza (by the EMA) |
| AF03 | Squalene; Tween-80; trometamol | Approved in pandemic influenza (by the EMA) |
| SE | Squalene; lecithin; block copolymer; glycerol; vitamin E | Clinical evaluation for *Leishmania,* influenza |
| CoVaccine | Squalane; sucrose fatty acid sulfate; Tween-80 | Clinical evaluation for hepatitis |
| EMA, European Medicines Agency; MDP, muramyl dipeptide; SE, stable emulsion. | | |

conventional influenza vaccines. FLUAD, the MF59-containing seasonal influenza vaccine that has been licensed in numerous countries for the past decade, has been shown to enhance the immune response in this nonresponder population.61 A similar candidate vaccine using the AS03 oil-in-water emul- sion has been tested in elderly adults for impact on influenza symptoms, demonstrating that the adjuvanted vaccine had advantages over the nonadjuvanted vaccine.62

**Enhancing the Breadth of the Immune Response.** Possi- bly one of the most important breakthroughs in adjuvant research during the last few years was the observation during the development of H5N1 pandemic influenza vaccines that oil-in-water adjuvants not only enhance the immune response and allow for dose reduction, but also enhance diversity and affinity of the antibodies induced.63 This qualitative and quan- titative expansion of the antibody repertoire has tremendous relevance for vaccination against pathogens, which undergo frequent antigenic drifts, such as influenza, as this would reduce the need for a perfect match between the antigen and the circulating pathogen strain.

This observation was first demonstrated in the ferret chal- lenge model with the AS03-adjuvanted H5N1 vaccine through cross-neutralizing antibodies and lethal challenge.64 The cross- neutralizing antibodies were confirmed later in clinical set- tings using the same AS03-adjuvanted H5N1 vaccine.65 Data published with MF59-adjuvanted H5N1 vaccine showed the induction of epitope spreading from HA2 to HA1 hemagglu- tinin and to neuraminidase, suggesting that this is a common feature of this family of adjuvants. MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines.66

**Mode of Action.** For many years oil-in-water emulsions were classified as vehicles, and it was assumed that the mode of action was primarily through enhanced delivery of the antigen to APCs or to the lymph nodes, even though most antigens do not associate physically to the oil droplets. It has, however, been shown that these emulsions stimulate the immune response indirectly. Using gene microarray analysis, Mosca and colleagues67 demonstrated that skeletal muscle fibers are the target of MF59, where the adjuvant induces production of PTX3 and JunB, which, in turn, stimulate pro- duction of TNF-α, IL-1B, and CCLs (chemokines C-C ligands), resulting in activation of resident APCs and recruitment and activation of circulating APCs. A similar mechanism has also been demonstrated for the α-tocopherol–containing oil-in- water emulsion, AS03. Morel and colleagues68 showed that similarly to MF59, AS03 induces expression of a range of cytokines, granulocyte recruitment at the injection site, and

increased antigen uptake by monocytes and migration to the draining lymph node. In this adjuvant, however, the expres- sion of some of the cytokines, such as IL-6, was modulated by the presence of α-tocopherol, which also enhanced the mag- nitude of the immune response, suggesting an immunomodu- latory action of α-tocopherol independent of the oil emulsion. The same authors also demonstrated that the adjuvant effect is local and that temporal and spatial colocalization of antigen and adjuvant were required; that is, injecting the adjuvant in a site distant to the antigen or at a later time resulted in no adjuvant effect, consistent with a local and short-lived direct impact of the immune system.

**Safety of Squalene-Containing Oil-in-Water Emulsions.** During the H1N1 pandemic, two influenza vaccines contain- ing an oil-in-water emulsion as adjuvant were approved and distributed in Europe (Pandemrix and Focetria); however, the uptake of these vaccines was hampered by broad media claims of the dangers of squalene. Many of these concerns seem to have arisen from a 2002 report that soldiers return- ing from the Gulf War with the so-called Gulf War syndrome had antisqualene antibodies that had been induced through receiving vaccines allegedly containing squalene.69 Although the vaccines in question did not apparently contain squa- lene, the WHO Global Advisory Committee on Vaccine Safety reviewed all available data, including data from clini- cal trials with the squalene-containing FLUAD vaccine. Animal studies have also suggested that squalene can induce arthritis.70 However, in the animal model used, the require- ment for a specific breed and the use of a complex protocol irrelevant to the vaccination practice make it difficult to assess the relevance of these data for safety evaluation in humans. The committee concluded that there was no evi- dence that squalene could induce pathological antisqualene antibodies. Before recommending the use of squalene- containing oil-in-water emulsion for the H1N1 pandemic, the WHO also reviewed all clinical data from more than 35,000 volunteers of all ages and concluded that there were no significant safety concerns.

Thorough safety surveillance by authorities during the

2009–2010 pandemic season showed a positive benefit-to-risk profile for the vaccines. Since August 2010 however, an increased number of cases of narcolepsy has been reported in children and adolescents vaccinated with an AS03-adjuvanted H1N1 pandemic vaccine in three northern European coun- tries. Interim reports of epidemiologic studies conducted in those countries have suggested an increased risk of narcolepsy in some vaccinated persons.71

The European Medicines Agency Committee for Medicinal Products for Human Use (CHMP) undertook a thorough

review of all available data as of July 2011, and concluded that the overall benefit-to-risk profile of the AS03-adjuvanted H1N1 vaccine remains positive. The CHMP acknowledged that further research in the areas of genetic and environmental factors in particular need to be explored before definitive con- clusions can be drawn. In October 2013, based on the safety and efficacy results obtained for the pandemic H5N1 vaccine, the H5N1/AS03 adjuvanted vaccine received a positive opinion from the Vaccines and Related Biological Products Advisory Committee (VRBPAC) for use in adults older than age 18 years.

Although it is not known how the adjuvanted vaccine could cause narcolepsy, several hypotheses have been pro- posed, including the potential role of viral proteins such as the nucleoprotein,72,73 suggesting that it is the presence of influenza viral nucleoprotein in the vaccine, rather than the adjuvant, that may be responsible. There are, however, no data clearly establishing a link between the adjuvant and the onset of the disease.

**Other Oil Alternatives for Squalene.** Little investigation into alternative metabolizable oils has been reported. As men- tioned earlier, squalane was used in the SAF emulsion and is included in the CoVaccine adjuvant; however, it is not clear whether this oil can be metabolized or whether it is elimi- nated through the skin.74 Miglyol, a metabolizable semisyn- thetic mixed triglyceride, has been evaluated as an oil-in-water emulsion adjuvant75 and was shown to enhance immunity but at lower titers than a nonmetabolizable mineral oil. In the future, synthetic oils may overcome some of the challenges associated with the use of animal-derived squalene.

## Toll-Like Receptor Agonists

Although TLRs and their role in triggering the innate immune response were understood only after their ligands were identi- fied as adjuvants, it is useful to classify these adjuvants through their specific receptor.

## TLR4 Agonists

Although LPS, a major component of the outer membrane of Gram-negative bacteria, has long been known to be a potent stimulator of the immune system, its use in adjuvants has been curtailed by its toxic effects. Early studies demonstrated that removing the core carbohydrate, generating lipid A, reduced the pyrogenicity without reducing the immune- stimulating activity. Lipid A was still too pyrogenic for use; however, it was shown that formulating lipid A in liposomes reduced the pyrogenicity a further 100- to 1000-fold, and lipo- somal lipid A formulations were used in clinical trials for candidate malaria vaccines without severe adverse events.76 In 1984, Ribi77 demonstrated that a significantly less-toxic mol- ecule could be obtained from LPS through sequential acidic and basic hydrolysis steps. The phosphate from the reducing- end glucosamine of lipid A derived from *Salmonella minnesota* RC595 is removed by mild acid hydrolysis, resulting in a mol- ecule, referred to as monophosphoryl lipid A (MPLA, referring to all other TLR4 agonists) that was significantly less toxic than the lipid A and that still stimulated the immune response.7 Ribi and colleagues then observed that if this MPLA was sub- jected to a further mild alkali hydrolysis, which selectively removed the acyl chain from the 3′ position of the disaccha- ride backbone, the resulting molecule, 3-deacylated MPLA (3d-MPL, or MPL), was even less pyrogenic but still exhibited adjuvant activity. GlaxoSmithKline developed a combination of adjuvants, AS04, in which MPL is formulated with alumi- num salt. The adjuvant system AS04 is used in two approved

vaccines (Cervarix [HPV vaccine] and Fendrix [hepatitis B vaccine for hemodialyzed patients]). Cervarix is aimed at pre- venting HPV infection and subsequent development of cervi- cal cancer. Extensive clinical trials demonstrated an acceptable safety profile and efficacy of the vaccine against the vaccine strains during a period of more than 8 years in clinical trial follow-up,78 as well as against divergent HPV strains not present in the vaccine.79

**Mechanism of Action of TLR4 Agonists.** Immune recogni- tion of TLR4 agonists, such as LPS or MPL, is initiated by extraction of monomers from the aggregates by LPS-binding protein in the serum.80 Monomeric LPS is transferred from LPS-binding protein to another accessory protein, CD14, which, in turn, transfers LPS to MD2, a secreted glycoprotein that associates with the extracellular domain of TLR4 to form the heterodimeric receptor that is responsible for the physio- logical recognition of LPS.81 Lipid A bound to the TLR4–MD2 complex activates two distinct intracellular signaling path- ways, which have come to be known by the names of the TLR4-proximal adaptor proteins, myeloid differentiation 88 (MyD88) and TIR domain-containing adaptor-inducing IFN-β (TRIF).82 The Myd88 pathway leads to the activation of mitogen-activated protein kinase– and nuclear factor-κB– dependent proinflammatory responses, whereas the TRIF pathway activates kinases responsible for type I interferon responses.83 [Fig. 6.1](#_bookmark0) shows this schematically. To this extent, the TLR4 receptor is unique among the TLRs by its ability to induce two distinct signaling pathways. The activation of these pathways is dependent on the structure of the agonist, where minor changes in the number and length of the acyl chains can have a major effect on pathway activation. MPL has been reported to activate the TLR4-TRAM (TRIF-related adaptor molecule)-TRIF–based signaling pathway and the TLR4-MAL (MyD88 adaptor-like)-MyD88 pathway.84

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**Species Specificity of TLR4 Receptor.** One challenge exists, however, in the development of TLR4 agonists for use in humans—the variability in the receptor specificity across species. Human, but not murine, TLR4–MD2 transmits proin- flammatory signals in response to hexaacylated but not pen- taacylated LPS.85 Moreover, in humans, but less so in rodents, tetraacylated and pentaacylated forms can inhibit the adjuvant activity. As a result, molecules shown to work in rodents will not necessarily function the same way in humans. One example of this is the OM-174 molecule, a triacylated mole- cule that functioned in mice,86 but apparently did not function in humans.

**Other TLR4 Agonists.** As MPL is isolated from a Gram- negative bacterium, *S. minnesota*, and such extraction can present manufacturing hurdles, many groups started to develop MPL analogs by chemical synthesis. [Fig. 6.3](#_bookmark11) illustrates the structure of MPL and its analogs.

RC529, a member of the aminoalkyl glucopyranosides developed by Corixa scientists to provide a synthetic alterna- tive to MPL, is structurally similar to a hexaacyl MPL, but the reducing terminal glucosamine has been replaced by a nonsac- charide backbone.87,88 This molecule, formulated with alum, is in a hepatitis B vaccine approved in Argentina (SUPERVAX) that is reported to increase immune response compared with nonadjuvanted vaccine.89

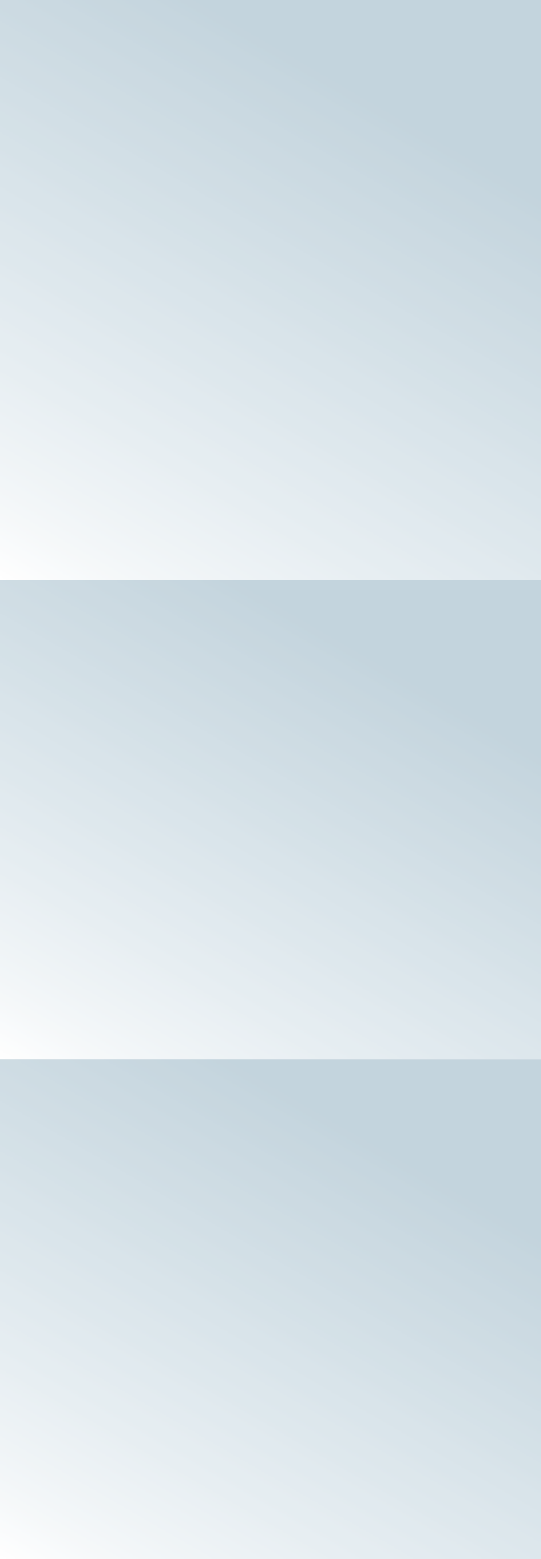
Glucopyranosyl lipid adjuvant (GLA) is a synthetic hexaa- cyl form of MPL, but it is designed on the *Escherichia coli* form of LPS rather than the *S. minnesota* LPS. The molecule has a single acyl chain on the 2′ amine and has an acyl chain on the 3′ hydroxyl.90 Clinical studies are in progress in different area such as tuberculosis (TB)91 and influenza.92



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| --- | --- |
| Structure | Name |
| Et2N OH  O  O O  (HO)2PO O HO O  O NH HO  O O NH OH  O  O O  O O  O  (C11)  (C11)  (C11) (C11)  (C13)  (C15) | MPL |
| OH  O O  (HO)2PO O Et2N O  O HN HN  O O  O  O O O  O O  (C14)  (C14) (C14)  (C14)  (C14) (C14) | RC-529 |
| O OH  O P O O O  NH4 HO O O  O NH HO O  O O O O NH  OH  O O HO O  HO  (C14)  (C14) (C14)  (C14)  (C14) (C14) | GLA |

|  |  |
| --- | --- |
| Structure | Name |
| O  HN NH  P P  HO O O O O OH  O HN \* NH O  O O  O O  \* O O O O \* | E6020 |
| O CH3OH  HO P O O O  HO HO HO O  NH OH  O HO NH P  O OH  O O O  O  HO | OM-174 |

**Figure 6.3.** Structures of TLR4 agonists in development or in licensed vaccines. GLA, glucopyranosyl lipid adjuvant; MPL, monophosphoryl lipid A.



E6020 is a synthetic TLR4 agonist that is not based on a saccharide backbone, but it is able to stimulate the TLR4–MD2 pathway.93 E6020 is in Phase I trials.

Butantan, the Brazilian vaccine manufacturer, has initiated a program to produce MPL from the LPS extracted from *Bor- detella pertussis*, a side product of the whole-cell pertussis vaccine process. This has been reported to enhance immunity to influenza vaccines.94 Heptaacyl sulphonyl sucrose, a com- ponent of the CoVaccine HT adjuvant, currently in clinical development, bears strong resemblance to the backbone struc- ture of TLR4 agonists and is thought to also act through TLR4.57 One question remains, however, concerning the ability of synthetic TLR4 agonists to overcome polymorphism associ- ated with the TLR4 receptor.95 Indeed, it is not clear whether all recipients will respond equally to a given TLR4 agonist molecule and whether a mix of various molecules, as present in the different naturally produced TLR4 agonists, will be necessary to compensate for human diversity.

**Formulation Challenges.** Ensuring an optimal and repro- ducible adjuvant effect requires some formulation knowhow. In the currently approved vaccines with TLR4 agonists (Cer- varix and Fendrix), the MPL is combined with an aluminum salt, and the combination of the two adjuvants is referred to as AS04. The combination of MPL with an aluminum salt adjuvant demonstrates some of the challenges of using MPL and analogous multiacylated disaccharide TLR4 agonists. Only a well-defined and controlled process can ensure that those molecules, which are insoluble in water, will not clump into aggregates that would lead to difficulty during sterilization by filtration or variability in the adjuvant activity. Various other approaches have been adopted to formulate TLR4 agonists, such as their incorporation into small unilamellar liposomes permitting a stable formulation. However, the activity is dependent on the ratio of lipid to agonist and how the agonist is combined with the liposomes.96 Other alternatives include the incorporation of the agonists into oil-in-water emulsions or application of thermal or sonic energy to form colloids, which can be further stabilized by the addition of small quanti- ties of lipids. The immune enhancement induced by TLR4 agonists is highly dependent on the physical structures, and determination of the consistency and stability of these requires detailed physicochemical characterization.97 Successful use of these adjuvants, therefore, requires careful consideration of how to formulate them and how these amphipathic molecules will interact with other components of the vaccine, such as surfactants, aluminum salts, and antigens.

**Safety of TLR4 Agonists.** MPL, by far the most widely used of the TLR4 agonists, has demonstrated an acceptable safety profile. A metaanalysis of data from 11 clinical trials and more than 74,000 volunteers (two-thirds of whom received the vaccine that contains AS04 and one-third the control) shows no increase in severe adverse events over aluminum alone or hepatitis A vaccine controls.78 It has been administered to mil- lions of young women worldwide with few reported severe adverse events. This was further supported by the mechanism of action of AS04, which demonstrated that the adjuvant effect is local and that temporal and spatial colocalization of antigen and adjuvant were required; injecting the adjuvant in a site distant to the antigen or several hours after injection of the antigen resulted in no adjuvant effect.13

RC529 (see [Fig. 6.3](#_bookmark11)), a synthetic TLR4 agonist in an

approved hepatitis B vaccine, has a limited history of clinical use, but as for MPL, no severe adverse events are associated with vaccine containing it. It is, however, not certain that other TLR4 agonists will have the same safety profile. Minor changes in the structure of TLR4 agonists can modify the way they

activate the MyD88 or TRAM–TRIF pathway, promoting type 1 interferon or proinflammatory responses, and, as described earlier, the difference in receptor specificity between rodent and human TLR4 receptors may prevent these being detected in preclinical models. The acceptability and extensive history of whole-cell pertussis vaccines, which contain residual bacte- rial LPS, suggests that this is a pathway that can be stimulated without long-term adverse effects.

## TLR9 Agonists

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Bacterial DNA is recognized by mammalian species as a PAMP and serves as a potent activator in the innate immune system through interaction with the intracellular TLR9. Fragments of bacterial DNA and synthetic single-stranded oligodeoxynucle- otides (ODNs) containing unmethlyated cytosine phosphate guanine (CpG) motifs (CpG ODNs) found in bacterial DNA have also been demonstrated to be powerful adjuvants.98,99 In vivo, however, the lability of phosphodiester bonds results in rapid degradation of DNA oligonucleotides, and it has been found that replacing these labile ester bonds in the oligonucle- otide sequences with phosphorothioate bonds stabilizes the oligonucleotides and significantly enhances the activity.100 Most preclinical and clinical studies with CpG-containing oli- gonucleotides contained this modification.

Although obtaining pure CpG-containing oligonucleotides is easy, the development of this class of molecules as adjuvants for human vaccines is severely hampered by the fact that the specific hexameric CpG motifs inducing optimal immune enhancement differ between species.98 Hence, evaluation of efficacy and safety in rodents is not readily translated to humans. Furthermore, the distribution of the TLR9 receptor and the pathways that its activation promotes also differ across species. In humans, CpG motifs are recognized by TLR9 found on natural killer (NK) cells,101 B cells, and plasmacytoid den- dritic cells,98 but are not recognized by myeloid dendritic cells and monocytes. In contrast, in mice, those cells express TLR9, which recognizes CpG motifs. In both species, CpG motifs trigger B-cell activation and induce directly or indirectly the production of Th1 and proinflammatory cytokines, including IL-1, IL-6, IL-18, TNF-α, and interferon-γ; in some cases, CpG can redirect preestablished Th2 responses toward Th1.102 CpG ODNs also act as adjuvants for antigens delivered by the mucosal route of administration.103

Numerous clinical evaluations of CpG as an adjuvant have

been performed, most commonly with a sequence of CpG referred to as 7909, a sequence found to be optimal for human use. CpG has been evaluated with and without alum for malaria vaccines,104,105 conjugate pneumonia vaccines,106 and hepatitis B vaccines107,108 and in HIV-infected patients in whom CpG permits rapid and long-term seroprotection.107 Further- more, it has been demonstrated in an elderly target population that when chemically conjugated CpG (1080 sequence) to the HBV (hepatitis B virus) antigen, the seroprotection induced was faster, superior, and more durable than three doses of a licensed comparator HBV vaccine (Engerix-B).109

CPG has also been used in cancer vaccines, such as non– small-cell lung cancer or melanoma, but as of this writing, with no significantly positive results in humans.110 In addition, an improved hepatitis B vaccine containing CpG (Heplisav B), which demonstrated higher and faster immune responses than the current alum adjuvanted vaccine, did not get a posi- tive opinion from VRBPAC (October 2013) because of the too-small size of the intended population safety database and was requested to extend it before potential licensure.

**Other TLRh Agonists.** TLR9 receptors were thought for a long time to be specific for unmethylated CpG sequences; however,

it has been shown that poly(dI:dC), a DNA version of the well- known TLR3 agonist poly(I:C), is also a potent adjuvant, acting through the TLR9/MyD88-dependent pathway.111 This oligo- nucleotide, in conjunction with a cationic peptide, is being developed as an adjuvant termed IC31 in clinical trials with recombinant TB antigens, and is reported to induce long- lasting T-cell responses to the antigens.112 The mode of action thought to be independent of IC31 is dependent not only on the poly(dI:dC) TLR9 agonist, but also on the interaction of the oligonucleotide with the coformulated cationic peptide. The complex formed entraps the antigen and acts as a physical depot at the site of injection, resulting in sustained antigen and adjuvant release.113 As for the examples given with TLR4, this demonstrates how critical formulation parameters are to suc- cessful adjuvant development

## Other Toll-Like Receptor Ligands

The molecules described in the preceding sections account for the vast majority of late-stage clinical trials and approved vac- cines containing adjuvants. However, numerous other TLR agonists are entering or are in early phase clinical trials. These are briefly described in the following paragraphs.

Flagellin is a protein that polymerizes to form the flagella of flagellated bacteria and is recognized by TLR5. This protein has been expressed as a fusion partner to influenza hemag- glutinin or influenza M2e, in which it serves as an adjuvant, enhancing the immune response to the influenza antigen as shown in clinical trials.114–116

Numerous small molecules have been found to act as ago- nists of TLR7 and TLR8. The best known of these are the small-molecule nucleoside analogs, imiquimod and resiqui- mod (R-848),117,118 that are TLR7 and TLR7/TLR8 ligands, respectively (both produced by 3M). Other well-known ago- nists include loxoribine119 and bropirimine.120 The use of these molecules as adjuvants is complicated by their low molecular weight and rapid removal from the site of injection. Several formulation methods enable these molecules to be used as adjuvants. Aldara cream, a 5% topical preparation of imiqui- mod that is licensed for treatment of genital warts and basal cell carcinoma, has been tested as an adjuvant for topical administration at the site of subcutaneous or intradermal injection of antigens.121,122 This has been shown to have some adjuvant activity in human clinical trials for cancer.123

## Other Adjuvants

Several other adjuvants are in advanced clinical development and do not fall into any of the aforementioned categories. The best known of these are the saponins, for which the mecha- nism of action is not fully understood. In this category, however, one could also include adjuvants such as virosomes, cationic liposomes, and polyelectrolytes, a class of polymers that includes polyoxidonium (a component of the Grippol influenza vaccine produced in Russia) and polyphosphazenes, which are being evaluated in several clinical trials.

**Saponins.** Saponins are triterpenoid molecules with a complex sugar backbone extracted from a variety of plants. The most widely used of these extracts is the saponin extract from the South American tree *Quillaja saponaria* Molina, referred to as Quil-A, which has been used as an adjuvant in veterinary vaccines since the 1970s. This mixture of saponins, with varying adjuvant activity and toxicity, was found to be too reactogenic for use in humans. The Quil-A saponins have an exquisite affinity for cholesterol, and when in contact with membranes containing cholesterol, they form a complex cre- ating pores in the membrane. At the site of injection, this

results in considerable reactogenicity. However, this affinity for cholesterol has also enabled the development of two adju- vants for human use based on the Quil-A saponins: ISCOMS and AS01.

ISCOMS are complexes of partially purified saponins from Quil-A, combined with cholesterol, to form small porous par- ticles 50 to 60 nm in diameter with a characteristic buckmin- sterfullerene shape and a high density.124,125 Originally, the antigen was associated with these structures through entrap- ment, conjugation, or hydrophobic interaction, processes that were tedious and not readily applicable to large-scale manu- facturing. Subsequently, it was shown that association of the antigen with the particle is not required, and simple coadmin- istration with antigen is adequate. As a result, simpler formu- lations (ISCOMATRIX and ISCOPREP), which can be simply mixed with the antigen, were developed by CSL (Austra- lia).126,127 These cholesterol–saponin complexes are less reac- togenic than the parent saponin, yet maintain a strong adjuvant effect. Clinical trials have shown them to be slightly more reactogenic than placebo or active control; however, the reactogenicity is generally mild and acceptable. No other vaccine-related severe adverse events have been reported.128

An alternative approach to reduce the toxicity of Quil-A

saponins was taken by Kensil and colleagues,129 who isolated from the mixture of saponins a pure component, termed QS21, that was less toxic yet retained adjuvant activity. QS21, however, had two drawbacks: It was chemically unstable at even mildly alkaline conditions, and, while less toxic than the parent mixture, it was still reactogenic. It was found that by combining QS21 with liposomes that contained cholesterol, the stability of the molecule was significantly enhanced and the reactogenicity abrogated.130 The adjuvant activity of this combination could be further enhanced by the addition of the TLR4 agonist MPL, and the resulting combination of adju- vants is termed AS01 by its developers, GlaxoSmithKline. This formulation has been tested in the clinical setting and shown to induce higher CD4 T-cell responses to a plasmodium antigen than the same immunostimulants combined with an oil-in-water emulsion.131 Further challenge studies demon- strated the higher efficacy induced by this formulation, which led to the selection of AS01 for the Phase III efficacy study of the candidate malaria vaccine.132,133 This again demonstrates the critical aspect of formulation and the challenges to iden- tifying appropriate formulations for optimal activity of adju- vants. QS21 in its pure form is also being used as an adjuvant for cancer vaccines.134

The exact mechanism of action of QS21 has not been fully

elucidated. The loss of adjuvant and lytic activity when the molecule is hydrolyzed suggests that membrane lysis has a role.129 However, the adjuvant activity is also lost when the aldehyde function on the triterpenoid backbone is removed.135 Several synthetic analogs have been developed that may permit a more detailed analysis of critical components of the molecule and a better understanding of its mechanism of action.136,137 In addition to the malaria vaccine, AS01 has been used as the adjuvant of choice for a herpes zoster vaccine, based on a recombinant antigen. The zoster vaccine demon- strated a reduced risk of shingles of 97.2% in adults age 50 years and older compared to placebo,138 and is the first recom- binant adjuvanted vaccine to demonstrate such a level of effi- cacy against disease reactivation in individuals with latent virus.

There is renewed interest in saponin-based adjuvants from

other plant sources, particularly from researchers in China and India.

**Virosomes.** Virosomes are reconstituted liposomes contain- ing viral (typically influenza virus) proteins in the liposomal

membrane and, optionally, with additional antigens incorpo- rated in the liposomal membrane or attached to the mem- brane.139 Virosomes have been used for influenza vaccines (licensed in Europe as Inflexal) and as adjuvants for hepatitis A vaccine (licensed in Europe as Epaxal). They are under inves- tigation as adjuvants for numerous other targets.140

**Polyelectrolytes and Polycations.** The influenza vaccine Grippol licensed in Russia contains polyoxidonium,141 a poly- electrolyte copolymer of N-oxide 1,4-ethylene piperazine and (N-carboxyethyl)-1,4-ethylene piperidium bromide, that has immunostimulatory properties.142 Even though little has been published about this specific polymer and its use as an adjuvant, an analogous polymer, poly(carboxylatophenoxy) phosphazene (PCPP) has been widely investigated as an adju- vant for vaccines and has been shown to exert adjuvant activity.143,144

Polycations such as polyarginine,145 chitosan,146,147 and cat- ionic lipids148 have also been shown to exert adjuvant activity. As for polyelectrolytes, the mode of action is not fully under- stood but is likely to involve interaction with cell membranes similar to that described for alum.32 Another cationic adju- vant, CAF01, is a liposome-based adjuvant composed of the cationic quaternary ammonium lipid dimethyldioctadecylam- monium (DDA) and the synthetic analog of mycobacterial cord factor; trehalose-dibehenate (TDB). CAF01 was originally developed as a CMI-promoting adjuvant for a subunit vaccine against TB, but the adjuvant has since been demonstrated to promote a diverse immune response resulting in CMI and humoral responses149,150 and is in clinical evaluation.

**Mucosal Adjuvants.** There is a strong interest in the vaccine community in administering vaccines via the mucosal route. This route offers the advantages of being easier to administer and not requiring trained health care workers, but also has the potential of inducing mucosal immunity at the portal of entry of many pathogens. While mucosal delivery and induction of mucosal immunity can easily be achieved with live attenuated vaccines (e.g., oral poliovirus vaccines, oral rotavirus vaccines, nasally administered live attenuated influenza vaccines), inducing systemic or mucosal immunity with nonlive antigens is much more difficult. These tend to be nonimmunogenic or poorly immunogenic via the mucosal routes; consequently, numerous adjuvants have been evaluated to enhance the immunogenicity.

The most widely investigated are the bacterial adenosine

diphosphate (ADP)-ribosylating exotoxins such as cholera holotoxin and *E. coli* heat-labile enterotoxin.151 Even though these are potent mucosal adjuvants, their toxicity has, for the most part, precluded their use as adjuvants. Consequently, mutations have been introduced in an attempt to reduce their toxicity while aiming to retain some adjuvant activity.152 These include adjuvants such as mLT(R192G), dmLT,153 and LTK63.154,155 For nasal delivery, these adjuvants have, however, presented unacceptable safety concerns. The native toxin was used as an adjuvant in an intranasal influenza vaccine that was licensed in Switzerland but was associated with occasional severe adverse events in the form of Bell palsy (partial facial paralysis) ascribed to the adjuvant,156 and similar effects were seen in an experimental vaccine using the LTH63 detoxified mutant.157 These adjuvants all contain the pentamer B subunit of the toxin, which binds ganglioside GM1 on nerves and can be transported through retroaxonal transport to the root of the nerve; it is thought that the neurological adverse events may have arisen through such an interaction.157 An alternative adjuvant, which does not contain the B subunit and, hence, does not bind GM1 gangliosides, is CTA1-DD, which is cur- rently in development.158,159 These exotoxins also have been

evaluated for oral delivery in several clinical trials; however, their susceptibility to acidic environments requires enteric for- mulations. The double mutant dmLT is currently being inves- tigated as an adjuvant for oral or sublingual vaccination against enterotoxigenic *E. coli* (ETEC).160

# FUTURE DIRECTIONS

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The past decade has seen an unprecedented surge in under- standing how adjuvants work. While the history of adjuvant discovery and development has evolved through serendipity, we are now in a position in which identification, selection, and use of adjuvants can be rationalized. This has opened the door to vaccine candidates against as yet unmet medical needs—vaccines that will not only prevent infectious diseases, but might also treat life-threatening diseases such as cancers or enable management of chronic disorders such as Alzheimer disease. Adjuvants appear also as a potential route to improve on current licensed vaccines that may be less efficient in a specific target population that requires a stronger stimulation on the immune system to develop a protective immune response.

There are a number of factors and unknowns that need to be considered. The most important of these is how to demon- strate the safety of adjuvants. As pointed out several times, there is an underlying concern that is not necessarily based on sound science that adjuvants can induce immune-mediated disorders. The absence of suitable animal models and the dif- ferences in receptors and receptor distribution between animals and humans can make it difficult in some cases to demonstrate that this is not the case.

Despite the occurrence of autoimmune reactions in the general population, the fear that adjuvants can induce or exac- erbate autoimmune disorders is one that is at the forefront of follow-up and surveillance. Holding clinical development of a vaccine based on a single adverse event demonstrates the challenges of developing vaccines with novel adjuvants. The risk-to-benefit balance associated with the vaccine being devel- oped will always be the guiding principle for ascertaining the value of a given approach. A large safety database to demon- strate rare events in such studies is not always possible, which underlines the difficulty introducing new approaches into the field of vaccines. The paucity of epidemiologic data on such immune-mediated disorders, which are key to establishing the background rate of disease, needs to be addressed so that factual analysis can be undertaken when such events occur.

The safety evaluation of a vaccine encompasses all constitu-

ents of the product. It cannot be assumed that an adjuvant that is safe in one vaccine with a given antigen will be safe when added to another vaccine, even if the latter vaccine is safe without adjuvant. A rational approach requires nonclini- cal toxicology, determination of the mode of action of the adjuvant, an evaluation of differences in receptors and activity in animal and human cells, controlled clinical trials, and post- marketing surveillance.161

Also, adequate formulation is critical for the activity of many adjuvants. Yet the knowhow for adjuvant formulation is not widely available, even though predicting how the physi- cochemical parameters of an adjuvant and its interaction with the antigen affect immune responses is key to its selection. All these points emphasize the criticality of process development, robustness, and reproducibility, and the ability to characterize adjuvants in a relevant and efficient way.

A host of adjuvants are now in clinical development; however, none tested in humans so far has the ability to induce functional CD8+ T-cells to a level that can be seen with live viral vaccines. Live viral vaccines have their limitations, in particular with respect to repeated boosting and their use in

immune-suppressed persons. There is, therefore, a need to pursue research into adjuvants capable of inducing CD8+ T-cell responses.

Finally, because vaccines are built on the combination of antigen(s) and adjuvant(s), the need for relevant and immu- nogenic antigens should not be overlooked. Because more and more vaccines will require the induction of both humoral immunity and CMI, there is a need for researchers to seek to improve the intrinsic immunogenicity of the antigen and to ensure optimum immune responses if the addition of an adju- vant is required.

It is only through the appropriate combination of antigen and adjuvant, selected on the basis of the targeted disease, relevant protective immune response, and target population, that adjuvants will fulfill their promises and find their place as a relevant and effective tool for improving human health.

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