Vaccines & Vaccine Technologies
New Generation Vaccines: Need for Safe and Improved Adjuvants

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Abstract

Vaccines are formulated to generate strong immune responses against administered antigens thereby providing long-term immunity, especially against microbial infections. To achieve this goal, safe and optimal vaccines are designed by developing new generation vaccines with efficacious adjuvants. These vaccines are mainly based on recombinant forms of protein subunits that are purified from molecular constructs of microbial agents with known chemical structures. However, these vaccines are poorly immunogenic and thus cannot induce an appropriate protective immune response. Therefore, to increase in vivo efficacy and stability of these vaccines, a safe and improved adjuvant is essential. Adjuvants in human vaccine formulations should be non-toxic, biologically inert, biocompatible, biodegradable as well as capable of facilitating the induction of antigen-specific immune responses. Currently, alum compounds are the only adjuvants approved in human vaccines. Though many clinically tested adjuvants more potent than alum are available, safety and regulatory concerns preclude their use for human applications. To address life-threatening pandemic diseases and ever evolving drug-resistant microbial infections worldwide, designing vaccines with safe and efficacious adjuvant is a major challenge for the scientific community that needs to be addressed systematically.

Keywords:
Adjuvant, Immune response; New generation vaccines; Vaccines

Introduction

Vaccines have an extraordinary role in medicine by improving human health many fold and have written a successful story by enhancing community health worldwide. Annually, vaccines are saving millions of humans from life-threatening diseases. Because of the successful vaccination procedures, morbidity and mortality rates are declined significantly. Microbes such as rotavirus, papillomavirus, and pneumococci kill millions of people worldwide and it is difficult to control them by old vaccines [1]. However, new vaccines are in progress and few of them are in their final stages of development. Most importantly, vaccines against infectious diseases such as Acquired Immune Deficiency Syndrome (AIDS), hepatitis, malaria, dengue fever and Leishmaniasis have not reached to the level of clinical use because of several factors. The major obstacles in designing these vaccines are microbe genome instability, antigenic variation, scarcity of funds, lack of appropriate animal models, absence of optimal adjuvants and lack of correlates of protection against diseases. Long time taken during clinical trials is another important factor that delays vaccine research and development. Generally, this process involves antigen selection, in vitro and in vivo efficacy tests, toxicology and animal evaluations and finally human phase I, II and III clinical trials. Since vaccines are given to infants and young children, who have no prior medical history, their safety is a primary concern. In this context, it is important to develop new adjuvants for vaccines that are safe and economically viable. This book chapter focuses on challenges we face today with new generation vaccines, current scenario of adjuvants research, safety and regulatory issues in the development of such new adjuvants for vaccine applications. Here, we used examples of vaccine formulations that were developed with various adjuvants against infectious diseases caused by Human Immunodeficient Virus (HIV), Hepatitis C Virus (HCV), malaria, influenza and dengue fever to explain the need for safe and improved adjuvants for new generation vaccines.

New Generation Vaccines and Challenges in their Development

In new generation vaccines, developing vaccines against HIV is the most important and highly demanding one. HIV causes significant morbidity and mortality around the world. More than 60 million are infected since the identification of the virus, while 33 million are living with AIDS and millions more are being infected daily [2]. Despite the intensive efforts to develop AIDS vaccine, rate of new infections is increasing exponentially thereby overwhelming disease prevention strategies. So far, only three vaccines reached up to the level of clinical phase trials [3]. First vaccine was based on gp120 monomeric protein combined with an alum adjuvant. However, this vaccine was ineffective in controlling infections due to lack of induction of protective antibodies against viral isolates [4]. A recombinant adenovirus based type 5 vaccine was the second AIDS vaccine, which was a combination of gag, pol and nef components of the virus [5]. This second vaccine also failed to control infections, although the cause and mechanism of failure of this vaccine needs to be explored in future. Third vaccine was based on prime boost strategy containing canary pox vector priming followed by a booster dose of gp120 antigen. This strategy provided initial positive signs of prevention, but the efficacy was only around 31% [6], which needs...
to be improved further. Clinical trials using these vaccines are still under progress. Vaccine development for HIV poses several scientific challenges including lack of availability of appropriate animal models, hyper variability of the virus, lack of correlation with protection immunity and virus integration into the genome of immune cells [3]. Apart from these obstacles, lack of an optimal adjuvant is also one of the important factors that impede HIV vaccine research and development. In this context, monophosphoryl lipid A (MPL) was found to induce Th1 type of immune responses through secretion of IFN-γ [7]. Recently, MPL was used in combination with QS21 saponin based adjuvant to enhance the potency of gp120 antigen in a clinical trial. However, this adjuvant formulation neither induced significant antibody response nor CTL responses [8]. In another study, MPL was found to induce antibody response with conjugates of HGP-30 (part of HIV Gag p17) and peptide segment of MHC II β chain [9]. QS21 is another potent adjuvant, which induced Th1 responses. In clinical trials with gp120, QS21 induced T cell proliferation but failed to induce the CTL responses [10]. Moreover this adjuvant induces moderate toxicity after injection. CpG is another Th1 inducing adjuvant and after formulation with gp120 antigen, it induced antibody, IFN-γ and CTL responses [11].

Similarly, various cytokine and chemokines such as IL-1, IL-12, GM-CSF were tested with HIV antigens. For example, combined adjuvant formulation of Flt 3 ligand and IL-1β induced Th1 and CTL responses to gag antigen [12]. In another study, intramuscular immunization of HIV envelope peptide with three different cytokine adjuvants IL-1α, IL-12 and IL-18 induced systemic and localized immune responses [13]. Apart from bacterial and cytokine based adjuvants, PLGA based nanoparticles have also been evaluated as an adjuvant during HIV clinical trials. Anionic PLGA particles easily adsorbed antigens on their surface and induced CTL responses [14]. When emulsion based SBAS-2 adjuvant was tested with gp120 envelope protein, induction of high antibody but not CTL responses was observed [8]. Hence, appropriate adjuvant formulation seems to be a pre-requisite for a vaccine to induce optimal immune responses against HIV infection.

HCV infections are another widespread disease that requires new preventive and therapeutic vaccines. One hundred and seventy million people are chronically infected with HCV and many more millions are continuously infected each and every worldwide. In 10-20% of chronically infected patients, cirrhosis was reported with a high risk for the development of liver cancer [15]. Although treatment appears closer, available new drug formulations are expensive and associated with severe toxic side effects. Therefore, development of an optimal vaccine is a challenge to control HCV infections, while minimizing liver cirrhosis and preventing hepatocellular carcinoma. Genetic variability is one of the major challenges associated with HCV infection [16]. HCV has a single stranded RNA genome, which transcribes and translates structural proteins E1 and E2 and, non-structural proteins; p7 ion channel, NS2-3 protease, RNA helicase, NS4A polypeptide and NS5B RNA dependent RNA polymerase [17,18]. The replication capacity with no proof-reading activity of RNA polymerase is the cause of such genetic variations [19]. Due to this variation, six different major types and hundreds of subtypes were identified worldwide [20]. The virus itself has the ability to mutate one nucleotide per replication cycle. In addition, both the envelope proteins E1 and E2 display greater level of variability. Moreover, amino terminal of the E2 protein contains few amino acid residues, which are having high degree of adaptation and this is known as first hyper variable region (HVR-1). HVR-1 plays an important role including virus binding and entry into the host [21]. Apart from this genetic variation, lack of an appropriate animal model and an efficacious adjuvant delay HCV vaccine development.

DNA vaccination is a potent strategy for treatment of HCV infection but its efficiency is dependent on appropriate adjuvant formulation. Different adjuvants and their combinations were studied to enhance potency of the DNA vaccine. Earlier, Karami and their colleagues tested aluminium phosphates, dendrosome, CpG and a combination of aluminium phosphates and CpG as adjuvants with the DNA vaccine. Among them, a combination of aluminium phosphates and CpG enhanced the efficiency of HCV core pcDNA vaccine [22]. A different lipid based adjuvant phosphatidylinositol mannoside (PIM2ME) was evaluated and compared with conventional adjuvants such as alum and MPL with the HCV antigen. Mice immunized with PIM2ME and HCV antigen induced higher cell mediated immune responses compared to controls. In vitro, PIM2ME also induced dendritic cells to produce IL-12 and IFN-γ [23]. Recently, adjuvant potential of IL-12 was analyzed with HCV NS3 DNA in mice. Co-administration of IL-12 with HCV NS3 significantly induced cytokines (IL-4 and IFN-γ) and proliferation of lymphocytes [24].

Generally, activation of Th1 response is needed for the development of a potent HCV vaccine. Hence, adjuvants inducing Th1 responses are better in promoting HCV vaccination. In this context, BCG as an adjuvant induced Th1 responses, when injected with HCV core antigen, which elicited significantly higher IFN-γ production and lymphocyte proliferation compared to polymer based F127 adjuvant. Total IgG and IgG1 subclass expression were also higher in BCG adjuvant group than Th2 response inducing F127 adjuvant [25]. Heat shock proteins (HSP) gp96 as an adjuvant also evaluated with HBsAg/HBcAg DNA vaccine. High antibody and T cell responses were observed against HBV infection compared to immununized mice, low serum HBsAg and viral DNA were observed in mice immunized with gp96 HBsAg/HBcAg DNA [26]. Moreover, layered double hydroxide (LDH) nanoparticles were also evaluated as an adjuvant with HCV DNA vaccine. These nanoparticles were efficiently taken up by the antigen presenting cells and, T cell proliferation and antibody responses were high in mice immunized with LDH nanoparticles loaded with HBV DNA [27].

Development of malaria vaccines is another intensive area of research and these vaccines are the most sought-after for humans. Five hundred million are infected by malaria each year worldwide, especially in Africa and Asia [28]. A deadly form of malaria is caused by the parasite Plasmodium falciparum, which is transmitted through highly widespread mosquito vectors Anopheles gambiae and Anopholes funestus. After decades of fighting against malaria, we still do not have any optimal vaccine. Though few vaccines are in the pipeline, a long time is still needed for these vaccines to come to the final stage. This might be attributable to reduced funding, complexity of malaria parasite life cycle, extensive antigenic variations and poor understanding of host-pathogen interactions [29]. One advantage with malaria vaccine development is the induction of clinical immunity after infection with the malarial parasites. Published evidence shows that persons who are infected by hundreds of mosquito bites each year acquire immunity early in their childhood against severe life-threatening complications [30] and adults develop clinical immunity more rapidly than children [31,32].

Alum is frequently used in malaria vaccines. Alum was tried at first in the evaluation of SP666 antigen but short-lived antibody response and weak cellular responses were observed [33]. PCS102 was another antigen, which was evaluated with alum adjuvant but this vaccine was also ineffective to protect against malaria [34]. Alum was moderately efficient with MSP1-C142 [35] and AMA1-C1 antigens [36] and induced good antibody response but with poor cellular responses. Currently, alum is frequently using with blood stage antigens such as EBA-175 RII [37], MSP3,151-225 and MSP3,151-225. These antigens with alum induced significant antibody responses and also inhibited the growth of malaria parasite under in vitro conditions [38,39]. GMZ2 is another recombinant antigen, which showed promising antibody response and induced adjuvant, which retained immune memory up to one year [40].

Besides alum, emulsion based MF-59 adjuvants were tested in clinical trials of malaria vaccines but they failed to provide protection
against malaria. For example, MSP-142 antigen formulated with MF-59 induced only low antibody titres and has no protection against malarial infections [41]. Similar results were observed in clinical trials of SERA-1 antigen with MF-59 adjuvants [42]. On the other hand, adjuvant AS03 showed only moderate protection (around 25%), but it elicited a strong antibody response with RTS,S vaccine in healthy individuals [43]. Several malaria vaccines are in clinical trials with ISA-720 montanide adjuvant. For example, ISA-720 montanide adjuvant with PICS102 antigen showed induction of functional antibodies and short-term memory responses [34]. Montanide adjuvants induce both humoral and cellular responses, but are associated with localized and systemic toxicities. Saponin (QS21) as an adjuvant was tried in a clinical evaluation of malaria antigens such as SP66. When SP66 was mixed with alum and saponin, it induced long-term cellular immune responses [44]. Besides these adjuvants, MPL was also used in the clinical trials of different malaria vaccines [45].

The development of new adjuvants is also highly required for the pandemic diseases like influenza. The influenza H5N1 strain is a highly pathogenic virus, which causes severe life-threatening disease leading to increased mortality. Vaccination efforts against influenza virus are complex in their implementation at large scale around the world and also, large and multiple doses are needed to achieve optimal vaccination effect. This is mainly because of the weak immunogenicity of the H5N1 strain [46]. At first H5N1 vaccine without an adjuvant was found to be poorly immunogenic. This vaccine required two doses of the antigen and hence several fold higher amount was needed against several endemic strains [47]. Later MF59 adjuvant based on emulsion (squalene in oil) was studied with H5N3 vaccine in sixty five human adults. It was demonstrated that mean titre value and sero-conversion rates were higher in MF59 vaccine treated compared to non-MF59 vaccine treated individuals [48]. Immunogenicity of flu vaccine varied with the kind of adjuvant used such as alum, MF59 or AS03. Also, it was demonstrated that alum showed low adjuvant potential than MF59 and AS03 adjuvants, irrespective of alum concentrations (300-1000 μg) used for vaccinations.

New adjuvants for influenza vaccines are designed to induce Th1, Th2 type cellular activation. In this context, cationic liposomes are promising [49] because it induced specific immune responses in clinical trials of influenza vaccines [50]. Besides liposomes, DNA motifs containing CpG induced immunity against influenza virus by activating TLR9 dependent signalling pathway [51,52]. CpG motifs induced both humoral and cellular immune responses and skewed immune responses toward Th1 pathway [53]. CpG DNA was also shown to increase the immunogenicity of influenza M2 antigen [54]. Besides CpG, other genetic adjuvants such as mycobacterium dependent protein-1 [55], heat shock protein (HSP 70) [56] and ESAT 6 [57] were also evaluated with avian influenza 5 DNA antigen. HSPs are chaperone proteins, which were used as genetic variants in influenza DNA based vaccines. Chaperones stabilize the DNA antigens and allow delivery to the dendritic cells [58]. Generally, these adjuvants induced CD8+ CTL responses, when fused with influenza antigens. For example, HSP-70 gene fused with H5 DNA of influenza virus was shown to be highly efficacious in inducing an immune response [55].

Dengue vaccines are other important highly demanded vaccines, especially in developing countries. Dengue is a viral disease with four distinct dengue virus serotypes (DENV 1-4) spread by mosquitoes and clinically it is characterized by a spectrum of illness starting from infection to non-specific febrile illness (dengue fever), dengue hemorrhagic fever and at last dengue shock syndrome [59]. Dengue is an endemic disease in many tropical and sub-tropical countries. Around 50 million are infected each year worldwide. Lack of satisfactory animal models mimicking the human disease and absence of an optimal adjuvant are major hurdles in the development of a vaccine for dengue fever [60]. Apart from the above-mentioned diseases, vaccines against rotavirus, Leishmania and other newly emerging drug-resistant microbes are major challenges for the scientific community. Overall, for the development of new generation vaccines against infectious diseases complexity of microbes, lack of appropriate animal models, funding scarcity, antigenic variations, genome instability and absence of an efficacious adjuvant are the challenges that need to be addressed systematically.

**History of Adjuvants and their Mode of Actions**

In 1926, for the first time, Glenny has demonstrated the adjuvant potential of alum compounds with diphtheria toxoid antigen [61]. This was followed by Freund’s experiments in 1937, who developed an adjuvant based on water in oil emulsion with or without mycobacterium component, known as Freund’s adjuvants [62]. Oil in water emulsion with mycobacterium is known as complete Freund adjuvant, while without this mycobacterium component is known as incomplete Freund adjuvant (IFA). Though complete Freund adjuvant (CFA) is toxic and causes keloid formation at the site of injection, it is still considered as a gold standard in immunology for immunizing rodents and primes for the development of disease models to study disease pathogenesis. Incomplete Freund adjuvant (IFA) is less toxic than CFA. IFA was used in some human vaccine formulations [63]. Of note, IFA induces an autoimmune polyarthritis disease in rats, the so-called adjuvant induced arthritis model [64]. During 1950s, adjuvant activity of lipopolysaccharides (LPS) was demonstrated [65]. Muramylidipeptide (MDP) is another microbial cell wall component (a peptidoglycan motif and NOD2 agonist) that was tested as an adjuvant in human vaccine studies around 1974 [66]. Adjuvant activity of several cytokines [67], unmethylated cytosine guanine dinucleotide (CpG) [68] were also tested in studies for developing human vaccines. Although various adjuvants more potent than alum are available, toxicity is the major concern that precludes their use in clinics.

In general, an adjuvant helps the administered microbial antigen (for example) to elicit strong antigen-specific immune responses that could contain or eliminate an infection. At physical level, adjuvants can release an antigen slowly for longer period of time, while at the cellular level their activation could be either via toll-like receptor pathways, inflammasome pathway or through direct activation of B and T cells (Figure 1) [69], leading to antigen-specific immune responses. Combination of both kinds of adjuvants can be used to design multi-component adjuvants for new generation vaccines. In vaccines, an adjuvant plays various roles such as elicitation of protective humoral and cellular immune responses, decreasing the dose of antigens needed and reducing the number of doses required to induce the long lasting immune response and, in the induction of mucosal immunity.
Figure 1: Schematic representation of adjuvants action and activation of the immune system. Adjuvant such as monophosphoryl lipid A (MPL) activate the immune system via toll like receptors (TLRs), while emulsion based adjuvants such as MF59 enhance the uptake of antigens through antigen presenting cells. Alum works via activation of inflammasome pathway.

Need for Safe and Efficient Adjuvants

During last 60-70 years, alum is the only adjuvant that was approved for human use. Alum is potent in enhancing immune responses to an antigen by activating Th2-mediated cellular mechanisms. Despite their good adjuvant potential, it is unable to induce other cellular immune pathways [70,71] and are often associated with toxicity problems [72]. Other restrictions with the use of alum adjuvants include increased IgE production [73,74] and neurotoxicity. Sometimes, low doses of alum can get deposited in kidney leading to reduced renal functions [75]. Occasionally, induction of autoimmunity is observed with vaccines containing alum adjuvants [76]. Moreover, alum compounds are unable to activate Th1-dependent cellular mechanisms, thus cannot be used in malarial vaccines [77], tuberculosis [78], cancer [79] and allergic diseases [80], where Th1 pathway of activation might be protective.

Vaccines are usually administered to healthy individuals including infants, children and adults; therefore safety is one of the major concerns with new adjuvants. Comprehensive preclinical studies are required to analyze biocompatibility and toxicity of adjuvants. According to World Health Organization (WHO), pre-clinical studies of vaccines should be done for their capacity to induce local inflammation and systemic toxicity before initiating clinical trials. In addition to this, at the early stage of a vaccine development itself the amount of adjuvant required in vaccine formulations need to be titrated at the individual level and compared with the vaccine without any adjuvant.

An Update on Clinically Tested Adjuvants

Conventionally, adjuvants such as alum compounds are widely used as adjuvants for human vaccines, which induce strong antibody responses against the administered antigens. Unfortunately, alum compounds are comparatively weak adjuvants and are incapable of activating cytotoxic T lymphocytes or Th1-type of immune responses [70,71]. Alum adjuvants work on the principle of depot generation for antigens at the injection site [81]. Apart from this, alum activates the complement system, eosinophils and, macrophages cells leading to enhanced uptake of antigens [82]. Calcium salts are another kind of mineral salts that are good in absorbing the antigens and adjuvant capacity of these salts was successfully demonstrated using diphtheria-tetanus-pertussis vaccine [83]. Calcium salts are less toxic than alum compounds and hence well tolerated in our body. Interestingly, calcium containing adjuvants are known to induce lower IgE production than the alum compounds and are capable of inducing significant levels of IgG antibodies [75].

Emulsion based adjuvants are another class of clinically tested vaccine adjuvants. Emulsions could be either oil in water emulsions.
such as montanide [84] or water in oil emulsions such as Freund’s adjuvant [62]. Similar to alum, emulsions also worked on the principle of depot generation for antigens to release them for a longer period of time. Although, emulsions are too toxic for human use still they are used as adjuvants for cancer vaccines, where a good acceptance of toxicity prevails to avoid mortality. The side-effects with emulsions include local inflammation and granuloma formation at the injection site [85].

Bacterial derivatives such a MDP [66] and LPS [65] are good mucosal adjuvants. Other compounds such as bacterial toxins and toxoids are other potential mucosal adjuvants that were tested earlier [86,87]. These adjuvants have great ability to activate cytotoxic T cell responses, apart from increasing the antigen presentation and differentiation of B cells into antibody secreting plasma cells through secretion of various cytokines [88]. Particulate adjuvants are another promising class of adjuvants, which mainly include liposomes [89], synthetic polymeric micro/nanoparticles [90], virosomes [91] and ISCOMS [92]. Particulates encapsulate greater amount of antigens and release them in a controlled manner, which is mainly attributable to their smaller size thereby enabling direct delivery and subsequent activation of antigen presenting cells. Adjuvant potential of liposomes mainly varies with the number of lipid layers, charge composition and synthesis protocol [89,93]. However, in vivo stability, toxicity and quality assurance are the major drawbacks with the particulate adjuvants.

Recently, researchers are trying a combination of two or more different adjuvants for minimizing toxicity, while enhancing the immunogenicity of an antigen. Generally, two or more adjuvants with different mode of actions can be combined. For example, alum was mixed with lipid A to enhance the immune potential of a vaccine [75]. In another example, alum was mixed with gamma-inulin resulting in algamulin formulation, which enhanced the adsorption capacity and stimulation of Th2 responses [94]. Similarly, immune-stimulatory complexes (ISCOMS), a combination of Quil A, lipids and cholesterol, acts as a virus particle with cage like structures in which several proteins can easily be encapsulated [95]. ISCOMS can be introduced into the body via different routes viz., oral, nasal and vaginal routes and can activate cellular immunity more prominently [95] but are associated with toxicity and instability problems.

Table 1 summarizes various clinical adjuvants and their combinations that are currently used for human vaccine formulations.

<table>
<thead>
<tr>
<th>Adjuvant class</th>
<th>Adjuvant</th>
<th>Immunogenicity</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral adjuvants</td>
<td>Alum compounds [61,70]</td>
<td>Potent adjuvant used in more than 80% of human vaccines, able to induce strong antibody responses</td>
<td>Toxic, can cause local inflammation at the site of injection, sometimes can induce autoimmune</td>
</tr>
<tr>
<td>Calcium phosphate [83]</td>
<td>Adjuvant for vaccines such as diphtheria, pertussis, tetanus and polio.</td>
<td>Potent adjuvant than alum as demonstrated with diphtheria toxin</td>
<td></td>
</tr>
<tr>
<td>Emulsions</td>
<td>Incomplete Freund adjuvant (IFA) [63]</td>
<td>IFA can induce antibody and DTH responses when administered with gp120 depleted HIV1 virus</td>
<td>Local and systemic toxicities. May cause local granulomas at the site of injection</td>
</tr>
<tr>
<td>Squalene oil in water emulsion (MF59) [48]</td>
<td>MF59 can induce secretion of different chemokines, enhanced antigen uptake by APCs and differentiation of dendritic cells</td>
<td>Local inflammation, granuloma formation at the site of injection</td>
<td></td>
</tr>
<tr>
<td>Montanide ISA-51 (water in oil emulsion) and ISA-720 [84]</td>
<td>Able to induce strong B and T cell responses</td>
<td>Local toxicities</td>
<td></td>
</tr>
<tr>
<td>Microbial components</td>
<td>CpG, synthetic oligonucleotides [68]</td>
<td>Activate Th1 pathways and work through TLR9 pathway as well</td>
<td>Mild inflammation at the injection site</td>
</tr>
<tr>
<td>Modified bacterial toxins such as heat labile toxin (LT), cholera toxin (CT) [86,87]</td>
<td>Bacterial toxins can increase serum and mucosal IgA secretion and activation of Langerhans cells</td>
<td>Local rashes with patch can be observed after immunization</td>
<td></td>
</tr>
<tr>
<td>Particulate adjuvants</td>
<td>Liposomes [89]</td>
<td>Can deliver antigens to MHC molecules, elicits weak antibody responses</td>
<td></td>
</tr>
<tr>
<td>Virosomes [91]</td>
<td>Good activator of antibody responses, through intranasal route and provide good protection against influenza virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymeric biodegradable microspheres [90]</td>
<td>They are capable of activating Th1 and Th2 responses in mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISCOMS (complexes of saponins and lipids) [92]</td>
<td>Can induce CTL responses with influenza antigens, worked via secretion of IFN-γ and IL-12</td>
<td>Can cause mild local inflammation</td>
<td></td>
</tr>
<tr>
<td>Combination of two or more adjuvants</td>
<td>AS04 (alum+monophosphoryl lipid A), AS02 (oil in water emulsion+MPL+QS-21), AS01 (liposome+MPL+QS21) [97]</td>
<td>Good mean antibody titre values, can induce CD8 T cell responses</td>
<td>Moderate local swelling and pain at the injection site</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>Polyinosine-polycytidylic acid [98]</td>
<td>Biased towards Th1 immunity</td>
<td>Stability and toxicity issues</td>
</tr>
</tbody>
</table>

### Mechanisms of Adjuvant Actions

Adjuvants are important for activating and enhancing the adaptive immune responses against vaccine antigens and their response can be mediated through B and T cells. Interactions of adjuvants with antigen presenting cells (APCs) cause secretion of several cytokines, which stimulate and differentiate B cells into memory B cells for long-term immunity and plasma cells for the production of antibodies against the delivered antigens. Adjuvants can also activate B cells through the activation of Th- cells (Th1, Th2 cells). IL-2 and IL-12 trigger Th1 cells, which secrete IFN-γ as the effector cytokine, while Th2 cells triggered by IL-4 secrete the effector cytokines IL-4, IL-5 and IL-13. IFN-γ activates macrophages as well as B cells for producing antibodies [69] and Th1-mediated cellular immune responses protect our body from intracellular pathogens. Th1 responses can also activate NK cells, which induces apoptosis in tumour cells; whereas, activation of the Th2 cellular immune responses lead to antibody responses against extracellular pathogens. Th17 cell mediated immunity triggered by IL-6 and TGF-3 comprises the effector cytokines IL-6, IL-1 and TNF-α, which are important against extracellular bacteria and fungi. Nasal mucosal immunization with a nano-emulsion was shown to induce both Th1 and Th17 immunity that was enhanced by TLR2 and TLR4 receptors [99]. Interestingly, the nature and extent of the Th- cell responses can be modulated by an appropriate adjuvant.

### Table 1: Different adjuvants used in human vaccines.
Currently, by using an appropriate adjuvant, immune responses can be modified that are biased towards either Th1 or Th2 cell signalling pathways. Thus adjuvants can be classified as Th1 or Th2 types. Th1 adjuvants include bacterial based components such as CpG, and MPL. Th1 adjuvants induce secretion of pro-inflammatory cytokines at the site of injection leading to recruitment of neutrophils, APCs and NK cells. Activated NK cells in turn secrete high IFN-γ production and drive the immune response towards Th1 pathway [100]. In contrast to Th1 adjuvants, injection of Th2 adjuvants such as alum induced low level of pro-inflammatory cytokines and limit recruitment of neutrophils. Therefore, Th2 adjuvants are associated with allergic reactions. For example, increased number of macrophages and monocytes were observed at site of alum injection [101].

Adjuvants show their effects in different ways. The adjuvant such as alum and emulsions (e.g. MF59, AS04) mainly act by depot effect for the encapsulated antigens at the injection site and release antigens slowly for longer period of time, which continues the activation of the immune system. These type of adjuvants enhance persistence of antigen at the injection site thereby increasing the infiltration of antigen presenting cells. Adjuvant such as alum can form multi-molecular aggregates with antigens and attract APCs at the site of injection [102]. Other adjuvants, such as microbial components act as ligands for pattern recognition receptors (PRRs) and induce innate immunity by targeting APCs. After interacting with PRRs, downstream signalling components such as transcription factors are activated leading to generation of effector cytokines. The secreted cytokines and chemokines play an important role in priming and expansion of the immune cells. Sometimes, interactions of an adjuvant such as DMD to NOD like receptors can also activate inflammasome pathway through secretion of IL-1β [5]. Few adjuvants can directly affect the antigen presentation by modulating the binding of antigens to major histocompatibility complex (MHC) molecules [102].

Toxicity and Regulatory Concerns of Adjuvants

Toxicity of adjuvants should be balanced against the benefits of incorporating them in vaccine formulations. Depending on the adjuvant components, toxic reactions could be either mild or severe. After injection of vaccine-adjuvant formulations local inflammation, necrosis, granuloma formation were reported at the injection site. Systemically fever, nausea, eosinophilia and allergy reactions can occur due to adjuvant toxicity. Unfortunately, potency of an adjuvant is directly correlating to their toxicity status. Familiar example is the complete Freund adjuvant, a potent adjuvant associated with severe local and systemic toxicities. Therefore, it is important to reduce toxicity of adjuvants, while evaluating the potency of the regulators concerning the use of adjuvants for human vaccines are exhaustively controlled than adjuvants applied for other purposes such as in animal vaccines. Hence, extensive pre-clinical studies are required to assess toxicity, while evaluating the efficacy and biocompatibility of a novel adjuvant.

Conclusions

Despite the intensive efforts to design optimal adjuvants for vaccinations over several decades, still we do not have any alternatives for alum compounds. New generation vaccines contain purified antigens that are based on recombinant technology and their further development requires efficacious adjuvants. Although more potent adjuvants than alum are available, associated toxicity issues and regulatory concerns preclude their use in human vaccines. Lack of understanding of the mode of action of adjuvants poses obstacles in designing new adjuvants. This is especially more important and an urgent issue in formulating vaccines for pandemic diseases such as influenza. Another main concern is that many potent adjuvants cannot be approved because though they are nontoxic when tested alone, in combination with a vaccine showed mild to severe toxicity effects. Lack of funding is another hurdle in the way of developing new adjuvants. However, new emerging drug-resistant microbial infections and life-threatening infectious diseases warrant development of safe and improved new adjuvants.

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