Human Vaccines & Immunotherapeutics 11:2, 477–488; February 2015; © 2015 Taylor & Francis Group, LLC

Advances in aluminum hydroxide-based adjuvant research and its mechanism

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Keywords: adjuvants,aluminum compounds,aluminum hydroxide,aluminum phosphate,immune response,inflammasomes,vaccine

In the past few decades, hundreds of materials have been tried as adjuvant; however, only aluminum-based adjuvants continue to be used widely in the world. Aluminum hydroxide, aluminum phosphate and alum constitute the main forms of aluminum used as adjuvants. Among these, aluminum hydroxide is the most commonly used chemical as adjuvant. In spite of its wide spread use, surprisingly, the mechanism of how aluminum hydroxide-based adjuvants exert their beneficial effects is still not fully understood. Current explanations for the mode of action of aluminum hydroxide-based adjuvants include, among others, the repository effect, pro-phagocytic effect, and activation of the pro-inflammatory NLRP3 pathway. These collectively galvanize innate as well as acquired immune responses and activate the complement system. Factors that have a profound influence on responses evoked by aluminum hydroxide-based adjuvant applications include adsorption rate, strength of the adsorption, size and uniformity of aluminum hydroxide particles, dosage of adjuvant, and the nature of antigens. Although vaccines containing aluminum hydroxide-based adjuvants are beneficial, sometimes they cause adverse reactions. Further, these vaccines cannot be stored frozen. Until recently, aluminum hydroxide-based adjuvants were known to preferentially prime Th2-type immune responses. However, results of more recent studies show that depending on the vaccination route, aluminum hydroxide-based adjuvants can enhance both Th1 as well as Th2 cellular responses. Advances in systems biology have opened up new avenues for studying mechanisms of aluminum hydroxide-based adjuvants. These will assist in scaling new frontiers in aluminum hydroxide-based adjuvant research that include improvement of formulations, use of nanoparticles of aluminum hydroxide and development of composite adjuvants.

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Submitted: 07/18/2014; Revised: 10/11/2014; Accepted: 10/23/2014
http://dx.doi.org/10.1080/21645515.2014.1004026

Introduction

Adjuvant (from Latin "adjuvare," meaning aid) is a substance that enhances immune responses through physical or chemical association with antigens. In particular, adjuvants assist in boosting specific immune responses against antigens contained in the vaccine.[1,2] In the past few decades, hundreds of materials have been tried as adjuvants. Examples include bacterial metabolites,[3,4] mineral oil/surfactant with immune-stimulant,[5] microparticles,[6,7] nucleic acids,[8] liposomes[9,10] and polysaccharide.[11] However, only aluminum based adjuvants continue to be widely used globally.[2,12]

A number of challenges had to be overcome to arrive at the current formulations of vaccines. Early formulations of vaccines were not pure. They were often contaminated with unrelated antigens that decreased the vaccine's efficacy. However, with the advent of recombinant DNA technology and synthetic chemistry, it is now possible to manufacture highly purified antigens to induce more specific immune responses. One major drawback of using formulations made from pure antigens is that they tend to have less immunogenicity. Therefore, such antigenic preparations require addition of an adjuvant to achieve protective immunity.[13] The diphtheria-tetanus-pertussis vaccine, the hepatitis A and hepatitis B vaccines, are examples of such vaccines that require the addition of an exogenous adjuvant to bolster the immune responses toward the antigens following immunization.[14] In contrast, certain other types of vaccine preparations contain endogenous adjuvants. For instance, vaccines that are manufactured from attenuated pathogens, such as the Sabin attenuated live polio vaccine, or killed pathogens, such as inactivated polio vaccine, contain endogenous adjuvants. A seemingly simple strategy of increasing the load of antigens in the vaccine to achieve desired immune response often results in adverse reactions. This is reflected in a study by Treanor et al. that investigated the safety and immunogenicity of an inactivated sub-virion influenza A (H5N1) vaccine in a dose-dependent manner.[15] The results of the study indicated that incidences of pain and tenderness at the site of injection were greater among vaccine recipients than placebo and the severity was clearly dose-dependent ($P < 0.001$). For such antigens, addition of adjuvant permits lowering of antigen content without compromising the immunogenicity conferred by the vaccine. Since its first use in 1932, billions of doses

of vaccines containing aluminum-based adjuvants have been successfully administered in humans, leading to a decrease in morbidity and mortality of infectious diseases. The widespread use of aluminum containing adjuvants can be attributed to their relatively lower cost and excellent safety. Although they may sometimes cause inflammation at the site of injection, they can also reduce the severity of systemic and local reactions by binding biologically active molecules in vaccines. Lastly, aluminum is found abundantly in our environment and is ingested daily through food and water, making it further suitable for use as adjuvant. [16]

In 1926, Glenny et al.[17] discovered that diphtheria toxoid (DT) precipitated with aluminum provided better immunogenicity than the toxoid alone. This pioneering study propelled the use of aluminum in vaccines as an adjuvant, a practice that has continued for more than 8 decades. Currently, aluminum based adjuvants are being used in vaccines like DTap, HepB and HepA. In addition to aluminum, recently, several new substances have been approved for use as adjuvants.[18] For instance, MF59 (Novartis Vaccines) is the first oil-in-water emulsion licensed for use as an adjuvant in humans and has been shown to enhance the host immune responses against homologous and heterologous inter-pandemic seasonal influenza viral vaccine strains in the elderly and other at-risk populations.[19-25] AS03 and AS04 produced by GSK,[26,27] CpG,[28,29] and poly-I:C[30,31] based adjuvant formulations are currently being evaluated in clinical trials. Although numerous studies have been published on potential of adjuvants in enhancing immune responses, in-depth studies on the mechanism of how adjuvants, in particular aluminum-based adjuvants, exert their function are lacking.

Aluminum based adjuvants used in vaccines mainly include aluminum hydroxide, aluminum phosphate and alum. Among these, aluminum hydroxide is the most commonly used chemical as adjuvant. The physical and chemical properties of aluminum hydroxide-based adjuvants and aluminum phosphate-based adjuvants are markedly different. These differences give rise to differences in immune responses evoked by these 2 chemicals.[32] Another notable difference between aluminum hydroxide-based adjuvants and aluminum phosphate-based adjuvants lies in their *in vivo* behavior. Flarend et al. studied[26]Al-labeled aluminum hydroxide-based adjuvant and aluminum phosphate-based adjuvant injected intramuscularly into rabbits over a 28 d period and found out that aluminum phosphate-based adjuvant dissolves more readily following injection.[33] This differential *in vivo* behavior affects the nature of immune responses evoked by the 2 adjuvants. Thus, different aluminum-based adjuvants elicit varied responses. This review is focused on discussing mechanisms of enhancement of immune responses by aluminum hydroxide-based adjuvants only because this adjuvant happens to be the most widely used form of aluminum as adjuvant.

Commonly used aluminum based adjuvants

Traditionally, aluminum based adjuvant vaccines have been prepared using 2 methods. The first method called the aluminum-precipitated vaccine method involves addition of aluminum containing suspension to a solution of antigen to form antigen-aluminum complexes. The second method referred to as the aluminum-adsorbed vaccine method entails addition of an antigen containing solution to previously prepared aluminum hydroxide, aluminum phosphate, aluminum hydroxide-aluminum phosphate mixture or alumina to form aluminum-adsorbed vaccine.

Since the aluminum hydroxide-based adjuvant is usually prepared by addition of alkali to the solution of aluminum salt to generate a crystalline aluminum oxyhydroxide [AlO(OH)],[34] the term "aluminum hydroxide-based adjuvant" does not reflect the actual chemical composition of the adjuvant. However, since the name "aluminum hydroxide-based adjuvant" has long been accepted and used for many years, in this review, we shall use this term to refer to the actual aluminum oxyhydroxide [AlO(OH)]. Larger assemblies of [AlO(OH)] may result via bridging intermolecular bonds between hydroxyl groups. Aluminum salt mixed with alkali form fluffy and flocculent aluminum hydroxide precipitate called crystalline aluminum metahydroxide. They form loose aggregates because of coordinated water. Aluminum hydroxide is an amphoteric compound with an isoelectric point of 11.4.[35] It carries positive charge on the surface in buffers with pH similar to the interstitial fluid of the body and can adsorb acidic protein antigens well.[36] Usually the particle size of aluminum hydroxide-based adjuvants prepared by different processes is heterogeneous. However, Alhydrogel® is an exception. This commercial preparation of vaccine has a relatively homogeneous diameter of the particles.[37]

Mechanism of immuno-stimulation by aluminum hydroxide-based adjuvants

The repository effect

After adsorption, antigens aggregate on the surface and inside aluminum hydroxide-based adjuvant particles, which helps in maintaining physical and chemical characteristics of the antigens. The adjuvant particles submit reposited antigens to the immune cells and promote interactions between antigens and immune cells for long durations to induce immune responses. This phenomenon is called the "repository effect" (also known as "depot effect").[2,38] Harrison verified the repository effect hypothesis by transferring the nodules formed by aluminum containing adjuvant precipitated toxoid from one guinea pig to a second guinea pig. [39]

The "repository effect" is mainly influenced by physical properties of aluminum hydroxide-based adjuvants such as surface area, electric charge, morphological structure, etc. Johnston et al. determined the surface area of a commercial preparation of aluminum hydroxide-based adjuvant by a gravimetric/FTIR method and obtained a mean value of 514 m^2/g.[40] Shi et al. found out that the specific surface area of aluminum hydroxide was enhanced at pH 7.4, 25°C which led to enhanced adsorption capacity that promoted antigen storage, interaction with antigen-presenting cells (APCs) and overall stronger immune response.[41] After injection of vaccine into organism, the antigen adsorbed on aluminum interacts with APCs, which primarily evokes an immune response. With the decomposition of aluminum

hydroxide, antigens inside aluminum hydroxide-based adjuvants are released gradually, which delays the consumption of antigen and prolongs the duration of stimulation of the immune system. If the interval of interaction between APCs and antigen is prolonged, a better immune response will result. The repository effect has been accepted as one of the mechanisms of aluminum hydroxide-based adjuvant's ability to stimulate immune responses for a long time.[42]

However, the repository effect alone cannot explain the mechanism of enhanced immune-stimulation by aluminum hydroxide completely. Several studies suggest that the antigen repository effect does not play an important part in adjuvanticity of aluminum hydroxide, and that aluminum hydroxide exhibits additional effects that account for its adjuvant properties. For example, Holt et al. injected diphtheria toxoid adsorbed with aluminum based adjuvant into guinea pigs and discovered that even if the tissue that had been injected by vaccine was cut off 7 d after inoculation, the effect of vaccination did not change.[43] Hutchison et al. reported that the removal of the injection site 2 hours after the administration of antigen/ aluminum containing adjuvant had no effect on antigen specific antibody and T-cell responses.[44] In addition, when Gupta et al. injected mice with tetanus toxoid, which had been labeled with ^{14}C and adsorbed on aluminum based adjuvant, the authors found out that the toxoid antigen was released promptly from adjuvant compounds.[45] Recent studies indicate that most antigens are able to release themselves from the surface of aluminum hydroxide-based adjuvants into the interstitial fluid, e.g. tetanus toxoid,[45] ovalbumin[46] and HIV-gp120.[47] Interestingly, this phenomenon also occurs in sheep lymph, which has similar characteristics as the interstitial fluid. Investigators infer that components of interstitial fluid (phosphate, citrate, fibrinogen, etc) can release antigen from the adjuvant.[18,48] These studies question the role for the repository effect during the course of vaccination. Data accumulated from studies on vaccines containing adjuvants so far are inconclusive about this mode of presentation of antigens to the immune system by the adjuvant. The interpretation of the mechanism of action of adjuvants is hampered by the nature of the experimental set-ups used in the studies. Both Harrison and Hutchison's studies lack an antigen-only control group, which is an important factor for comparison with the effect of adjuvanted group.[39,44] Hutchison stated that removal of injection site did not alter the magnitude and kinetics of antigen-specific immune responses following aluminum-based adjuvant containing vaccine immunization in mice.[44] However, in mice injected with antigen + CpG/aluminum adjuvants, the IgG2a response did appear to be partially dependent on the injection site being intact. Results of above studies seem to indicate that as long as the concentration of antigen at the site of injection is high enough and the antigen is engulfed by APCs, the repository effect is not strictly needed for aluminum hydroxide-based adjuvant. However, the repository effect may ensure high antigen concentration and enhance the process of uptake of antigens by APCs, which further bolsters immune responses.

Pro-phagocytic effect

Uptake of antigens by APCs is pivotal for induction of immune responses. Antigens adsorbed on aluminum hydroxide as well as those released into interstitial fluid can both be captured by APCs. Aluminum hydroxide in combination with antigens forms particles, which contribute to uptake by APCs.[49] Seema et al. studied the importance of interactions between interstitial fluid and adsorbed antigens following administration of aluminum hydroxide-based adjuvant-containing vaccines.[50] For all 3 proteins studied, immune-potentiating effect in the presence of aluminum hydroxide-containing adjuvants was observed. Ovalbumin and de-phosphorylated α casein desorbed rapidly in interstitial fluid, while α casein remained adsorbed when exposed to interstitial fluid. The authors inferred that ovalbumin and de-phosphorylated α casein were primarily taken up via pinocytosis, while α casein was primarily engulfed by phagocytosis. Rimaniol et al. investigated interactions between aluminum hydroxide and macrophages *in vitro* and discovered that macrophages carrying aluminum hydroxide exhibited distinct changes in their phenotype and function.[51] These changes resembled and had classical features of myeloid dendritic cells. They could induce MHC II type antigen-specific memory responses. These results demonstrated that macrophages are sensitive to vaccines with aluminum hydroxide-based adjuvant. Such vaccines activate macrophages to enhance immunological memory and confer long-term protection. Mannhalter et al. compared the uptake of tetanus toxoid when administered with or without aluminum hydroxide-based adjuvant using radiolabelled ^{125}I tracer experiments.[49] Labeled toxoid was incubated with macrophages *in vitro*. Aluminum hydroxide-based adjuvants significantly accelerated the speed of uptake of diphtheria toxoid. From 10 min - 6 h post incubation, the speed of uptake of tetanus toxoid by macrophages, in presence of aluminum hydroxide-based adjuvant, increased at least 5 folds. Three hours post injection, the uptake speed of antigens by the macrophages increased 10 folds compared to the group without aluminum hydroxide-based adjuvant. Thus, adjuvants promote phagocytosis that enhances immune responses against antigens.

Aluminum hydroxide-based adjuvants and NLRP3 pathway

Aluminum hydroxide-based adjuvants can recruit hemocytes, promote dendritic cell (DC) differentiation and accelerate local inflammatory reactions independently of Toll like receptors (TLR). However, the cellular target for unleashing the pro-inflammatory activity of aluminum hydroxide-based adjuvant remained unidentified until recently. Recent reports from different labs suggest that the aluminum hydroxide-based adjuvants target nucleotide binding oligomerization domain (NOD) like receptor protein 3 (NLRP3, also named as NALP3).[14,18,38,52] Li et al. reported that macrophages are mainly responsible for phagocytosis and processing of antigens. Aluminum hydroxide-based adjuvants activate endogenous-cellular immune responses mediated by NLRP3 and promote macrophages to secrete high-levels of pro-inflammatory factors such as IL-1β and IL-18.[53] This phenomenon is abrogated in cells lacking NLRP3 inflammasome components.[54] Studies of Kool et al.[54] suggested that

aluminum hydroxide-based adjuvant took part in innate and acquired immune responses evoked against ovalbumin (OVA) through the activation of the NLRP3 inflammasome.

NLRP3 is a member of NOD like receptor (NLR) family that undergoes oligomerization via caspase activation and recruitment domain (CARD). CARD interacts with aspartate protease 1 to form inflammatory corpuscles. After proteolytic activation *in trans*, inflammatory corpuscles modify precursors of pro-inflammatory cytokines (including IL-1β and IL-18), forming mature forms of these cytokines. *In vitro* studies reveal that aluminum hydroxide is able to activate aspartate proteases through NLRP3.[53,55] Interestingly, reactive oxygen species may be generated and lysosomal damage may appear in cells after endocytosis of aluminum hydroxide particles. Both these signals are upstream activator signals of NLRP3 inflammatory corpuscles. Moreover, aluminum hydroxide mediated cytotoxicity may further induce apoptosis of cell, resulting in release of trioxypurine, which can activate formation of NLRP3 inflammatory corpuscles indirectly. Studies by Eisenbarth et al. support a role for NLRP 3 inflammasome in the adjuvant effect of aluminum hydroxide-based adjuvants, and that the innate inflammasome pathway could direct a humoral adaptive immune response.[56]

Lambrecht et al. have discussed mechanisms of currently used aluminum hydroxide-based adjuvants.[57] In their studies, a clear increase in uric acid, an endogenous danger signal, was observed following injection of OVA in conjunction with aluminum hydroxide-based adjuvants in the peritoneal cavity of mice. Based on *in vitro* as well as *in vivo* experimental results, uric acid derived from necrotic and damaged cells at the injection site activated the NLRP3 inflammasome in a pathway requiring phagocytosis and promoted innate immune response.

Kool et al. reported that the stimulatory effects of aluminum hydroxide on cellular and humoral immunity were completely abolished when $CD11c^+$ monocytes and DCs were conditionally depleted during immunization.[58] DC-driven responses were abolished in MyD88-deficient mice and after uricase treatment, which implied a need for induction of uric acid for activation of immune responses. The authors suggested that aluminum hydroxide-based adjuvant is immunogenic by exploiting "nature's adjuvant," the inflammatory DC through induction of uric acid, the endogenous danger signal.

Kuroda et al. found that particulates such as aluminum hydroxide salts could activate the inflammasome and induce the secretion of proinflammatory cytokines in macrophages.[59] These particulates could also induce the production of immunoglobulin E via a T helper 2 (Th2) cell-associated mechanism.

Contrary to former studies, more recent studies on NLRP3 deficient mice vaccinated with aluminum hydroxide-based adjuvants revealed that deficiency of NLRPs had no significant effect on T and B cell responses. Therefore, the exact role of NLRP3 pathway in immuno-stimulatory effect of adjuvants remains unclear.[59,60] Evidence from results of several independent studies accumulated so far suggests the involvement of the NLRP3 inflammasome as well as other NLRP3 inflammasome-independent pathways in the mechanisms of aluminum hydroxide-based adjuvants, which are mediated through antigen presenting cells and subsequently, act directly or indirectly upon B and T cells.[61]

Aluminum hydroxide-based adjuvants and innate immune responses

Investigators have tried to identify cells targeted by aluminum hydroxide-based adjuvants for stimulation of immune responses by conducting *in vitro* experiments. Aluminum hydroxide-based adjuvants act on macrophages and not TLRs. They mediate their differentiation into DCs and enhance the ability of macrophages to submit antigens instead of activating DCs directly.[51] Moreover, aluminum hydroxide-based adjuvants play a role in the recruitment of hemocytes (inflammatory monocytes) to the site of injection. Aluminum hydroxide-based adjuvants can also facilitate the differentiation of inflammatory monocytes into DCs, which is consistent with *in vitro* results. Interestingly, inflammatory monocytes recruited by aluminum hydroxide express higher levels of MHC II due to significantly improved capacity to adsorb antigens. Antigen-carrying DCs differentiated from inflammatory monocytes can efficiently migrate to draining lymph nodes and induce intense T cell proliferation.[18]

Wang et al.[62] performed stage III clinical trial for therapeutic hepatitis B vaccine and surprisingly found out that therapeutic effect emerged in control group that was immunized with aluminum hydroxide-based adjuvant alone. Results of experiments conducted using transgenic mice indicated that sera TNF-α levels are elevated in groups immunized with adjuvant and hepatitis B vaccine. Aluminum hydroxide-based adjuvant group showed an increase in IL-10 expression also, which indicates that aluminum hydroxide-based adjuvants can induce inflammatory responses, which will then lead to exertion of therapeutic function. Jordan et al. identified in mice a previously unknown population of IL-4-producing $Gr1^+$ cells, which after injection with nitrophenyl-conjugated bovine serum albumin and the commonly used aluminum hydroxide-based adjuvant, could lead to the secretion of IL-4, followed by the priming and proliferation of splenic B cells and their accumulation in the spleen.[63] The same effect was found in mice injected with aluminum hydroxide-based adjuvant alone, which suggested that this effect of aluminum hydroxide-based adjuvant was antigen independent.

Marichal et al. reported that in mice, aluminum hydroxide-based adjuvant caused cell death and the subsequent release of host cell DNA, which acted as a potent endogenous immunostimulatory signal mediating aluminum hydroxide-based adjuvant activity.[64] The authors also proposed that host DNA-evoked immune stimulation could differentially regulate IgE and IgG1 production after aluminum hydroxide adjuvanted immunization. These examples illustrate the ability of aluminum hydroxide based adjuvants to boost innate immune responses upon administration with antigens.

Aluminum hydroxide-based adjuvants and acquired immune responses

It is generally accepted that the stimulation of immune system through TLR is the premises for initiation of T cell dependent

immune responses because this stimulation leads to complete maturation of DCs and co-stimulates signal transfer to T helper cells. Studies on MyD88 knock-out mice showed that stimulation of B cells through TLRs is necessary for T cell-dependent antibody production.[18] Researchers found out that aluminum hydroxide-based adjuvants are unable to directly activate DCs and thus make them express co-stimulatory molecules and release pro-inflammatory cytokines *in vitro*. This suggests that aluminum hydroxide doesn't activate TLR dependent signaling pathways. Experiments using double-mutant mice lacking MyD88 and TRIP revealed that synchronous immunization of aluminum hydroxide-based adjuvant and T cell-dependent antigens induce intense antibody production independently and without the requirement of TLR signaling pathways. Some studies show that acquired immune responses can be elicited without the participation of TLR signaling pathways. Aluminum hydroxide-based adjuvants may also act independently of TLR signaling.[65]

Flach et al. reported that aluminum hydroxide-based adjuvants could bind DC plasma membrane lipids with substantial affinity independent of inflammasome and membrane proteins.[66] Subsequent lipid sorting activated an abortive phagocytic response that led to antigen uptake. Such activated DCs showed high affinity and stable binding with $CD4^+$ T cells via the adhesion molecules intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1 without further association with aluminum hydroxide-based adjuvant. These results indicated that aluminum hydroxide-based adjuvants trigger DC responses by altering membrane lipid structures and suggest an unexpected mechanism for how this crystalline structure interacts with the immune system and how the DC plasma membrane may behave as a general sensor for solid structures.

Complement activation by aluminum hydroxide-based adjuvants

Ramanathan et al. investigated the ability of aluminum hydroxide compounds that cause granuloma formation and macrophage damage to activate the complement pathway and found that aluminum hydroxide compounds could activate complement in a way that did not necessarily involve either the classical or the alternative pathways.[67]

Main factors that influence effect of aluminum hydroxide-based adjuvants

Adsorption rate

The antigen adsorption ratio is one of the key factors that influences immune responses. Aluminum hydroxide-based adjuvants adsorb antigen through multiple physical and chemical interactions that include electrostatic attraction,[50] hydrophobic interactions[68] and ligand exchange.[69] Electrostatic attraction is the most universal mode of adsorption. Ligand exchange between hydroxyl group on aluminum and phosphate group of antigen has also been observed.[70-73]

Strength of the adsorption

The adsorption ability of aluminum hydroxide-based adjuvants is defined by 2 important parameters, 1) The capacity of adsorption, which provides information about the maximum quantity of antigens adsorbed by aluminum hydroxide; 2) The strength of adsorption, a parameter expressed by adsorptive coefficient, which can be calculated by applying an adsorption equation.[74]

Recent studies indicate that the degree of adsorption of antigen in the interstitial fluid following administration is directly related to the effectiveness of a vaccine. Chang et al. vaccinated rabbits with lysozyme based vaccines with *in vitro* adsorption degrees of 3%, 35% and 85%, respectively, and observed similar levels of immune responses in all the 3 vaccine groups.[75] To explain this unexpected result, they used sheep lymph fluid to simulate *in vivo* environment (interstitial fluid) that the vaccine encounters following subcutaneous or intramuscular injection. Three vaccines with different *in vitro* adsorption degrees were diluted with sheep lymph fluid. After 60 min, the degrees of adsorption of these vaccines were all transformed to 40%. These results demonstrated that immuno-stimulatory effect of aluminum hydroxide-based adjuvant is irrelevant to the adsorption degrees of aluminum hydroxide-based adjuvants *in vitro*, but the adsorption degree *in vivo* is an important consideration.[75] However, adsorption degree of antigen to aluminum hydroxide-based adjuvants *in vitro* indicates the consistency of vaccine manufacturing processes, and is therefore still an important quality control factor for final product.

Immuno-stimulatory effects may differ for same antigen adsorption degrees if the adsorption strengths (interactions between the antigen and the adjuvant) are different. Bethany Hansen et al. used four vaccines with different adsorption coefficients *in vitro* for vaccination in mice and found out that the antibody titer had an inverse relationship with the adsorption coefficient.[76] Adsorption strength of aluminum hydroxide-based adjuvant is affected by the concentration of the phosphate radical present in the vaccine. Junnan Tian et al. investigated the relationship between phosphorus content and immuno-stimulatory effect of recombinant hepatitis E vaccine with aluminum hydroxide-based adjuvant and concluded that the maximum adsorption rate of antigen to adjuvant was curtailed with an elevation of the level of phosphate radical in the vaccine. Junnan Tian et al. also found out that the adsorption rate of antigen in sheep lymph decreased in presence of phosphate while the antibody titer was up-regulated, which is consistent with the finding that higher concentrations of phosphate reduced antigen adsorption and enhanced antibody titer.[77]

Size and uniformity of aluminum hydroxide particles

Recent studies have shown that particle-size distribution and uniformity of $Al(OH)_3$ particles can affect the immuno-stimulatory effect of aluminum hydroxide-based adjuvants.[78] Huai et al. immunized NIH mice (weighing 10–14 g) with diphtheria toxoid adsorbed on 2 different sizes of $Al(OH)_3$. After 5 weeks, estimation of the antibody titers indicated that the vaccine adsorbed with $Al(OH)_3$ with mean diameter of 200 nm was superior to

that containing $Al(OH)_3$ particles of 600 nm diameter. In addition, the adjuvant with smaller particle size had better physical characteristics and absorption efficiency.[79] Ye et al. reported that adjuvants prepared by mixing $AlCl_3$ with NaOH were different in many characteristics like turbidity, diameter, uniformity and sedimentation than adjuvants prepared by mixing $AlCl_3$ with $NH_3.H_2O$. When adsorbed with HBsAg, vaccine prepared by mixing $AlCl_3$ with NaOH had a larger particle size than those prepared by mixing $AlCl_3$ with $NH_3.H_2O$. After immunization in mice, vaccine made from $AlCl_3$ and NaOH showed higher adsorption rate and immune efficacy ($P < 0.05$).[80]

Dosage of aluminum hydroxide-based adjuvant

The content of aluminum hydroxide-based adjuvant in each dose of vaccine is of paramount importance for eliciting optimal immune responses. Low content of adjuvant cannot adsorb the available antigen in entirety and therefore cannot induce immune responses effectively. Although sometimes smaller dosage may be enough to adsorb antigens completely, immune stimulatory effects should also be considered while selecting the amount of adjuvant to be administered. High aluminum hydroxide content can suppress immune reactions because it can suppress the release of the antigen. Using appropriate amount of antigens, an aluminum dosage-dependent effect on antibody production can be observed at a certain range of aluminum hydroxide-based adjuvant. High aluminum hydroxide content can also lead to cytotoxicity in phagocytic cells.[81] The commonly used dose of aluminum hydroxide-based adjuvant is 0.5 mg/dose (based on aluminum ion content). The aluminum hydroxide-based adjuvant content recommended by WHO is $\leq$1.25 mg of aluminum ion per dose. Zhang et al. screened different dosages of aluminum hydroxide-based adjuvant in their study on avian influenza vaccine (split virion) and found out that 1.2 mg/dose was the optimal dosage, which had highest neutralizing antibody titers in BALB/c mice and guinea pigs while conferring satisfactory immunity.[82] Thus, determination of the amount of aluminum hydroxide-based adjuvant to be added to a vaccine is a critical step in the overall vaccine production process.

Characteristics of antigens

The efficacy of vaccines containing aluminum hydroxide-based adjuvant is also dependent on the characteristics of antigens present in vaccines. Li et al. reported that aluminum hydroxide showed better adjuvant effect than aluminum phosphate, and inferred that this might be due to better adsorption with some proteins at neutral pH.[83] Shakhshir et al. suggested that different adsorbabilities of aluminum adjuvants were possibly due to differences in surface charges of adjuvants and proteins. For adsorbed compounds with low protein content, the surface charge of adjuvant will prevail. For adsorbed compounds with high protein content, the surface charge of protein will prevail.[35] Because of the diversity of immune responses induced by different antigens, immune responses induced by different antigen-adjuvant combinations may be even more varied. Vaccines for extracellular pathogens, bacterial exotoxin and intestinal parasites should be aimed at inducing Th2 immune responses, while vaccines for intracellular pathogens should be designed based on eliciting specific immune responses. Knowing the physical and chemical characteristics of $Al(OH)_3$ based adjuvant are not enough to predict the immuno-stimulatory effect or stability of vaccines, the surface charge characteristics of the antigen-adjuvant compounds after adsorption should also be considered.

Drawbacks of aluminum hydroxide-based adjuvant

After being in use for nearly a century, the processes for manufacture and application of aluminum hydroxide-based adjuvants have become mature. More than a billion doses of aluminum hydroxide adsorbed vaccines like DTP and hepatitis B vaccine have been safely injected in adults and children. However, aluminum hydroxide-based adjuvants have been found to have some drawbacks. For instance, inoculation of aluminum hydroxide-based adjuvant vaccine can cause local adverse reactions such as erythema, subcutaneous nodules, contact hypersensitivity and granuloma.

In a seminal study published in 1998, Gherardi et al. described a new inflammatory muscle disorder of unknown cause characterized by a distinctive pathological pattern of macrophagic myofasciitis (MMF).[84] Muscle biopsy showed infiltration of the subcutaneous tissue, epimysium, perimysium, and perifascicular endomysium by large macrophages with a finely granular Periodic Acid-Schiff stain (PAS)-positive content.[85,86] The chemical components of inclusions in macrophages from MMF patients were shown to be aluminum hydroxide-based compounds.[87] MMF was once thought to be an adverse reaction caused by intramuscular injection of vaccines because many intramuscular vaccines contained aluminum hydroxide-based adjuvants. Several studies have concluded that aluminum hydroxide-containing vaccines can lead to local tissue damage with symptoms similar to MMF when injected intramuscularly. MMF-like transient damages were also observed in experimental animal models that were injected with vaccines with aluminum hydroxide based adjuvant intramuscularly.[88]

Allergic reactions include another critical adverse drug reaction (ADR) of aluminous adjuvants. Firstly, acidophilic cells could be attracted by adjuvants to the site of injection, which in turn could lead to the increase in total IgE levels. These induce IgE-mediated allergies, which could potentially increase the sensitivity of susceptible individuals. Studies on guinea pigs by Sun et al. showed that $Al(OH)_3$ adjuvant at concentrations $\leq$4 mg/ml resulted in no allergic reactions. However, adjuvant concentrations of 7 and 10 mg/ml led to strong allergic reactions, and adjuvants at concentrations of 13 mg/ml induced the most intense reactions. Guinea pigs receiving 1.5 and 4 mg/ml of $Al(OH)_3$ exhibited no allergic reactions in passive cutaneous anaphylaxis test. These results set a limit of 4 mg/ml of $Al(OH)_3$ per dose as safe.[89] Secondly, aluminum hydroxide-based adjuvants could also act as a kind of antigen and elicit immune responses. Allergic reactions are primed at first injection, and hypersensitivities set in after the second injection. However, very few studies support this point of view.[90]

Another drawback of aluminum hydroxide-based adjuvant is that they cannot be stored frozen. Antigens in vaccines with aluminum hydroxide-based adjuvant are adsorbed and supported by the grid structure of aluminum salt, which is prone to destruction when frozen. Therefore, aluminum hydroxide-based adjuvant vaccines cannot be stored below zero degree Celcius.[91-96]

There are studies that are in disagreement with the drawbacks of use of aluminum hydroxide-based adjuvants. Theeten et al.[97] compared immunogenicity of DTP containing different concentrations of aluminum hydroxide-based adjuvant. Within a certain range of dosage and aluminum hydroxide-based adjuvant content, no significant increase in adverse reactions such as fever, redness and swelling were observed between different study groups. In fact, researchers even found out that adsorption and slow release of vaccine components may sometimes reduce the incidence and severity of local/systemic reactions. Norimatsu et al.[98] studied *in vivo* effects of aluminum hydroxide-based adjuvant on systemic reaction of bacterial lipopolysaccharide (LPS) in animal. Results showed that the lethality in mice group injected with LPS added to aluminum hydroxide gel was significantly reduced. Results of Shi Y et al.[41] revealed that aluminum hydroxide-based adjuvant detoxifies endotoxin by adsorbing it in the vaccine and slowing down it's releasing into interstitial fluid upon administration. Jennifer Hawken et al.[99] reviewed studies on adjuvants and Inactivated Polio Vaccine and stated that aluminum hydroxide-based adjuvants could enable a 3- to 4-fold dose reduction of IPV. Berthold et al.[100] studied the effect of $AlPO_4$ and $Al(OH)_3$ on the induction of antibodies against purified recombinant protective anthrax antigen (anti-rPA antibodies) in mice, and found that there was no significant difference between the anti-rPA antibody levels induced by 15, 7.5, and 3.75 μg of rPA in presence of aluminum hydroxide-based adjuvants, which indicated that adsorption enhances immunogenicity of lower doses of antigen. Lowering antigen use in vaccine could not only reduce the cost of antigen manufacturing, but also, more importantly, reduce adverse effects caused by antigens in vaccines. These examples clearly illustrate the beneficial effects of aluminum hydroxide based adjuvants.

Whether aluminum hydroxide based adjuvants can stimulate T-cell responses is not clearly understood. Traditionally, researchers tend to infer that Th2-type immune responses are preferentially primed by aluminum hydroxide-based adjuvants.[14] HogenEsch et al.[16] reviewed that aluminum hydroxide-based adjuvants selectively stimulate a Th2 immune response in mice and a mixed response in human beings. However, the authors have concluded with a cautionary note that recent studies on mechanisms underlying the immune-stimulatory effect of aluminum hydroxide-based adjuvants were mostly carried out using intraperitoneal injections in inbred strains of mice, and the relevance of these studies to the mechanisms of immune response of aluminum hydroxide-based adjuvants injected intramuscularly in human beings still remains to be determined. Our studies on hepatitis B vaccines suggest that both Th1 and Th2-type immune responses can be primed by aluminum hydroxide-based adjuvants injected intramuscularly. Hu et al.[101] evaluated cellular immunity in adults who were intramuscularly vaccinated with recombinant hepatitis B vaccine (rHB) produced in yeast and found out that IFNγ secreted by $CD8^+$ and $CD4^+$ T cells could be detected shortly after vaccination with stable level. He et al.[102] detected IFNγ, IL-2 and TNF-α levels by Luminex method in BALB/c mice（$H\text{-}2^d$）subcutaneously immunized with recombinant HB vaccines derived from 3 different expression systems and found that the IFNγ and TNF-α levels of mice induced by these vaccines reached peak values 10 d after immunization, while the IL-2 level increased gradually and reached peak levels at day 25–35. In another study, BALB/c mice were first immunized subcutaneously. A follow up booster dose containing equal amount of hepatitis B vaccine or recombinant hepatitis B antigen was administered. Serum samples were collected 24 h, 48 h and 7 d after administration of the booster dose for analysis of the cytokines secreted. IP-10, IL-12, p70, IL-5 and IL-6 were secreted at higher levels by vaccine group compared to antigen group (unpublished data). Wang et al.[103] evaluated the effect of aluminum hydroxide-based adjuvant on cellular immune responses induced by newly developed inactivated enterovirus 71（EV71）vaccine in mice. After subcutaneous immunization with aluminum hydroxide adjuvant-containing and adjuvant-free inactivated EV71 vaccines, respectively, levels of IFNγ, IL-6 and IL-10 secreted by both the study groups were estimated. Higher levels of cytokines were secreted by adjuvant-containing group when compared to adjuvant-free group. This suggests that aluminum hydroxide-based adjuvant can enhance Th1 and Th2 immune responses to inactivated EV71 vaccine. These results demonstrate that with appropriate vaccination route, aluminum hydroxide-based adjuvants can improve both Th1 and Th2 cellular responses to antigen.

New research directions of aluminum hydroxide-based adjuvants

Improvement of formulations

Theeten et al.[97] compared immune effects of DTP vaccine containing different concentrations of aluminum hydroxide and found out that there was no significant difference between 0.133 mg/dose and 0.5 mg/dose in eliciting immune responses against diphtheria and tetanus. No significant differences were found in seroconversion rates in pertussis antibodies. These studies suggest the possibility of decreasing aluminum hydroxide-based adjuvant content without compromising the effectiveness of the vaccine. Thus, optimization of amount of aluminum hydroxide is an important consideration for vaccine formulations.

The level of immune responses evoked by vaccination varies and is largely dependent on genetic/species background, antigen dosage, administration route, detection method, time, etc. Antigens prepared from same gene sequence, but by using different expression systems can elicit different responses. For example, Diminsky et al.[104] compared composition, structure and immunogenicity of recombinant hepatitis B surface antigen particles produced by mammalian cells (CHO) and yeast cells (*Hansenula polymorpha*). Differences were found in peptide and lipid

compositions of these 2 antigens. HBsAg produced by CHO cells (CHO-HBsAg) induced lower cytotoxic T lymphocyte response than HBsAg produced by yeast cells (yeast-HBsAg). Similarly, Hu et al.[105] evaluated the kinesis of cellular and humoral immune responses to 3 different kinds of recombinant hepatitis B vaccines in immunized mice, and found out that immune responses induced by these vaccines were different in their patterns and levels. Based on the intensity of early cellular immune response, the 2 yeast-based HB vaccines (*Hansenular polymorpha* and *Saccharomyces cerevisiae*) were superior than the CHO-based vaccine. Interestingly, CHO-based vaccine induced early seroconversion and highest level of anti-HBs. These results demonstrate that components from expression systems have great influence on antigen's reactivity. Hence, in vaccine research and testing, selection of expression system for the production of recombinant antigen is an important consideration.

Modification of aluminum hydroxide-based adjuvant

$Al(OH)_3$ adsorbs acidic proteins under physiological pH. However, it is a poor adsorbent for basic proteins. This inability has limited the range of its application. The characteristic properties of $Al(OH)_3$ can be altered by changing the composition of buffer solutions.[106] Rinella et al.[36] reported that the ζ potential of commercial $Al(OH)_3$ adjuvant was 26 mV in pH 7.4 buffer, which can be reduced to a negative value by increasing the concentration of phosphate group in buffer to $\geq$2 mmol/L. This study implied that processing of $Al(OH)_3$ adjuvant in presence of phosphate group can lead to transformation in charge. As a result, the $Al(OH)_3$ based adjuvant can adsorb basic proteins by electrostatic attraction. In presence of 5 mmol/L phosphate, the ζ potential of $Al(OH)_3$ changed to −16 mV, and the adsorption rate of lysozyme (pI 11.1) to $Al(OH)_3$ increased from 11% to 39%. Studies by Liu et al.[70] also demonstrated increased adsorption of antigens in presence of additional phosphate groups in malarial vaccines. However, external phosphate groups can interfere with the adsorption of phosphate group-containing antigens to $Al(OH)_3$ adjuvant by competing with phosphate groups in antigens, and lowering the efficacy of $Al(OH)_3$. Further research is required to find new chemicals that can substitute phosphate groups for modulation of charge of $Al(OH)_3$ adjuvant that could increase the repertoire of antigens that could be used with this adjuvant.

Nanoparticulate aluminum hydroxide-based adjuvants

Compared to traditional aluminum hydroxide-based adjuvants, adjuvant at nanoscale with same amount of aluminum hydroxide can adsorb more antigens because of smaller particle size, much larger specific surface area, higher surface reactivity, and stronger adsorption capacity. In 1981, nanoparticles of polymethylmethacrylate were first used as adjuvant in influenza vaccines, which could protect mice from murine influenza virus. They also offered improved thermostability.[107] He et al.[108] prepared a novel formulation of nanoparticulate (NP) alumimun hydroxide-based adjuvant specifically in the cationic water-in-oil micro-emulsions of water/benzalkonium bromide (BB) and octyl alcohol/cyclohexane at 30°C. After injecting intra-peritoneally into guinea pig, serum antibody titers of the first and second week after immunization estimated by ELISA were higher in NP group than the traditional aluminum hydroxide-based adjuvant group ($P < 0.01$; $P < 0.05$).

He et al. analyzed in-house preparation of aluminum hydroxide-based adjuvant by transmission electron microscopy (TEM) and differential scanning calorimeter (DSC) and confirmed that the resultant particles in the adjuvant were $Al(OH)_3$ crystals with a spherical shape (mean diameter: 72.62 nm). Serum anti-HBsAg IgG titers of nanoparticulate aluminum hydroxide-based adjuvant group were higher than those of regular aluminum hydroxide-based adjuvant group in BALB/c mice in the first and second weeks after immunization ($P < 0.01$; $P < 0.05$). These results highlight the ability of nanoparticulate aluminum hydroxide-based adjuvant to further enhance immune responses induced by HBsAg and elicit an early humoral immunity when compared to regular aluminum hydroxide-based adjuvant group.[109]

Tang et al. proved that nano-$Al(OH)_3$ particles could induce anti-AIV H_9 humoral immune responses without any side-effects earlier than traditional formulations of vaccine.[110] Moreover, in 2008, Tang et al. compared vaccines containing nano-$Al(OH)_3$ or traditional $Al(OH)_3$ based adjuvant and found nano-$Al(OH)_3$ particles aided Newcastle disease virus vaccine in inducing stronger humoral and cellular immunity against Newcastle disease virus in chicken.[111] The nano-$Al(OH)_3$ adjuvant was thermostable and could withstand sterilization at 121°C for 30 min. The characteristics of the adjuvant remained the same after the sterilization process, and therefore sterilization of adjuvant can further ensure the safety of vaccines.[112] Based on accumulated research results, vaccines with nano-$Al(OH)_3$ adjuvant can stimulate antibody production earlier than traditional vaccines and enhance the differentiation of Th cells to Th1 cells, which leads to more intense cellular immune response and facilitates the induction of rapid immune responses and clearance of virus. The homogeneity of nano-adjuvant makes antigen particles encapsulated or adsorbed by nano-adjuvant desirable targets of DCs and macrophages, which can greatly promote potent immune responses. Compared to conventional aluminum hydroxide-based adjuvant, nano aluminum hydroxide-based adjuvants significantly mitigate excessive inflammatory reactions (e.g., subcutaneous granuloma) at injection site.

Although nano aluminum hydroxide-based adjuvant has many advantages over traditional aluminum hydroxide-based adjuvants, recent studies have questioned the biosafety of nano materials. Shavedova et al. reported lung injury caused by single-walled carbon nanotubes.[113] Hussain et al. found out that nanocrystalline metal and metal oxide can induce renal and hepatic injuries.[114] Tsuji et al. found out that metal particles <200 nm in size could cause cerebral injury since they can cross the blood-brain barrier.[115] The accumulation of nano-TiO_2 in the brain tissue of mice could affect the metabolism of monoamine neurotransmitters.[116] Although current studies reveal tremendous potential of nano-$Al(OH)_3$ based adjuvants in eliciting potent, selective immune responses, further systematic studies on safety, toxicology and pharmacology are warranted to justify the use of nano-$Al(OH)_3$ based adjuvants.

Composite adjuvants

In order to overcome the shortcomings of aluminum hydroxide-based adjuvants and to evoke more potent immune responses, researchers have looked into development of composite adjuvant vaccines. These vaccines contain, in addition to aluminum hydroxide, other ingredients. AS04 (GlaxoSmithKline Vaccines), is a composite vaccine approved for use in humans for protective immunity against HBV (Fendrix).[117] AS04 can induce local NF-κB activity and cytokine production transiently, which leads to an increased number of activated Ag-loaded DCs and monocytes in lymph nodes at the site of injection. This in turn leads to an increase in the activation of Ag-specific T cells. HPV (Cervarix) vaccine is another example of a composite vaccine.[118,119] This vaccine is a mixture of 3-O-desacyl-4'-monophosphoryl lipid A (MPL, a TLR-4 agonist) and aluminum hydroxide-based adjuvant. Aluminum hydroxide prolongs the cytokine responses to MPL at the site of injection, while the addition of MPL to aluminum hydroxide enhances the response to vaccine by rapidly triggering a local cytokine response leading to an optimal activation of APCs.[120] In another study, Zhao et al. immunized mice with a mixture of BCG-CpG-DNA, recombinant HBsAg and aluminum hydroxide-based adjuvant.[28,121] Antibody and CTL tests indicated that BCG-CpG-DNA promoted the production of antigen specific IgG2a, induced Th1 immune response, and partially reversed Th2 response. Thus, composite vaccines seem to be effective and hold promise. As a result, the number of studies on composite adjuvants is on the rise. However, the immune enhancement effects of different composite adjuvants are complex and difficult to evaluate. Subtle variations like change in adjuvant dosage or adjuvant/antigen ratio for the same kind of composite adjuvant could alter the type of immune response desired (e.g., humoral or cellular response). Therefore, a number of parameters need to be tested to evaluate the efficacy of a composite adjuvant.

Conclusions

In this review, we have summarized results of recent research focused on the mechanisms underlying aluminum hydroxide-based adjuvants' ability to modulate immune responses. Clearly, this adjuvant employs more than one mechanism, which is conceivable since several different aspects of the immune system are affected. While some of the mechanisms have been studied in depth and are well supported by experimental evidences, others have conflicting evidences. For example, aluminum hydroxide-based adjuvants can facilitate the uptake of antigens by APCs. This implicates and firmly establishes a role for the adjuvant in modulating both innate as well as adaptive immune responses. On the other hand, the repository effect that was recognized as an important consequence of administration of aluminum hydroxide-based adjuvants in the past is now being questioned by several new reports. Inflammatory responses play an important role in immuno-stimulatory effect of adjuvants; yet whether aluminum hydroxide-based adjuvants act via NLRP3 inflammatory corpuscles, remains unclear. Release of DAMPs such as uric acid after aluminum hydroxide salt-induced inflammation is a recently discovered new mode of induction of innate immunity. However, this mode of activation is probably not the only method employed by aluminum hydroxide-based adjuvants to stimulate innate immune responses. Interpretation of effects contributed solely by aluminum hydroxide-based adjuvants from the published results is hampered in part by the differences between study designs, the formulation of adjuvants/vaccines, antigens used, and differences in animal models employed for conducting the studies. Moreover, most studies published are focused on investigating a role for antigens or vaccines (i.e., antigen + adjuvant) and not on evaluating the effect of aluminum hydroxide based adjuvants alone. A majority of the studies use aluminum hydroxide-based adjuvant solely for the purpose of "control group." More comprehensive studies revolving around the adjuvant are required to delineate the molecular mechanisms underlying its function. For example a study aimed at obtaining a structural view of aluminum hydroxide bound to NLRP3 followed by structure-guided mutagenesis studies could conclusively support and explain the mechanism of activation of innate immune responses by this adjuvant.

Most of the studies on aluminum hydroxide-based adjuvants have focused on limited factors and lack systemic analysis. The effect of all factors in combination needs to be evaluated. With rapid advances in immunology and a continuous increase in our knowledge of host-pathogen interactions, mechanisms underlying the ability of aluminum hydroxide-based adjuvants to modulate immune responses will become increasingly clear. Integration of technology could further speed up this quest. Already advances in bioinformatics have increased accuracy of prediction of a vaccine's effectiveness. For instance, using systems biology approach, researchers managed to predict the efficacy and immunogenicity of yellow fever vaccine and seasonal influenza vaccine at an early stage of development by identifying early gene "signatures." Each antigen has its own unique characteristics, and the interactions between antigen and aluminum hydroxide-based adjuvant in different vaccines may vary based on the kind of antigen, vaccine formulation, animals/species used, etc. Even for antigens expressed from same gene sequence, immune effects may differ because differences in manufacturing processes, vectors, and formulas affect the antigenecity. Thus, emerging technologies, new breakthroughs in the field of immunology and development of new methods will not only aid in elucidation of the molecular mechanisms underlying immune responses evoked by vaccines and their enhancement by adjuvants, but will also help identify desirable traits. A better understanding of the mechanisms and desirable traits will enable us to modulate the humoral and cellular responses to aluminum hydroxide adjuvanted antigens in order to develop more potent and less toxic vaccines.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors are grateful to Professor Li Jin of Beijing Tiantan Biological Products Co., Ltd. for reading and text correction of the manuscript.

Funding

This work was supported by a grant from the National Science and Technology Key Projects on "Major Infectious Diseases such as HIV/AIDS, Viral Hepatitis Prevention and Treatment" (2012ZX10004701).

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