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Aluminum compounds as vaccine adjuvants

Rajesh K. Gupta*

Wyeth-Lederle Vaccines and Pediatrics, 401 North Middletown Road, Pearl River, NY 10965, USA

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Abstract

Aluminum compounds are the only adjuvants used widely with routine human vaccines and are the most common adjuvants in veterinary vaccines also. Though there has been a search for alternate adjuvants, aluminum adjuvants will continue to be used for many years due to their good track record of safety, low cost and adjuvanticity with a variety of antigens. For infections that can be prevented by induction of serum antibodies, aluminum adjuvants formulated under optimal conditions are the adjuvants of choice. It is important to select carefully the type of aluminum adjuvant and optimize the conditions of adsorption for every antigen since this process is dependent upon the physico-chemical characteristics of both the antigens and aluminum adjuvants. Adsorption of antigens onto aluminum compounds depends heavily on electrostatic forces between adjuvant and antigen. Two commonly used aluminum adjuvants, aluminum hydroxide and aluminum phosphate have opposite charge at a neutral pH. The mechanism of adjuvanticity of aluminum compounds includes formation of a depot; efficient uptake of aluminum adsorbed antigen particles by antigen presenting cells due their particulate nature and optimal size (< 10 μm); and stimulation of immune competent cells of the body through activation of complement, induction of eosinophilia and activation of macrophages. Limitations of aluminum adjuvants include local reactions, augmentation of IgE antibody responses, ineffectiveness for some antigens and inability to augment cell-mediated immune responses, especially cytotoxic T-cell responses. © 1998 Elsevier Science B.V.

Keywords: Alum; Aluminum hydroxide; Aluminum phosphate; Adsorption; Adjuvanticity

Contents

*Corresponding author: Tel.: +1 914 7325320; fax: +1 914 7325490; e-mail: rajesh_gupta@internetmail.pr.cyanamid.com

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1. Introduction

Aluminum adjuvants have a long history of use with routine childhood vaccines which was founded on the discovery that a suspension of alum precipitated diphtheria toxoid had much higher immunogenicity than the soluble toxoid [1]. Aluminum compounds, including aluminum phosphate ($AlPO_4$), aluminum hydroxide ($Al(OH)_3$) and alum precipitated vaccines, historically referred to as protein aluminate, are currently the most commonly used adjuvants with human and veterinary vaccines [2–6]. These adjuvants are often referred to as 'alum' in the literature, which is misleading, because (1) alum, chemically potassium aluminum sulfate ($KAl(SO_4)_2.12H_2O$), has not been used as an adjuvant; (2) aluminum hydroxide and aluminum phosphate have different physical characteristics [7] and differ in their adjuvant properties [5]. Alum was originally used to partially purify protein antigens, mainly tetanus and diphtheria toxoids by precipitating them in the presence of anions including phosphate, sulphate, and bicarbonate ions resulting in a mixture of compounds, mainly aluminum phosphate and aluminum hydroxide [6,8,9]. The amounts of aluminum phosphate and aluminum hydroxide in the mixture depended upon the amount and nature of anions present in the reaction mixture and adjustment of pH of the final product with sodium hydroxide [5,6,10,11]. Although alum-precipitated tetanus and diphtheria toxoids had been used for human immunization for many years, their use has declined considerably due to variations in production of alum precipitated toxoids [3,6,8,9,12].

2. Method of preparation

Since aluminum compounds are the only adjuvants used routinely for human vaccines, these have become the benchmark or reference for evaluating new adjuvant formulations. It is very important to prepare optimal formulations of vaccines adsorbed onto aluminum adjuvants to correctly evaluate the new adjuvants. Two methods have commonly been used to prepare vaccines and toxoids with aluminum compounds - in situ precipitation of aluminum compounds in the presence of antigen, and adsorption of antigen onto preformed aluminum gel [2,5–7,9,13]. Adsorption of antigens on aluminum adjuvants depends upon physical and chemical characteristics of antigen, type of aluminum adjuvant and conditions of adsorption [5,7,11–15]. These conditions are often overlooked and a poorly formulated aluminum adjuvant preparation does not give optimal adjuvanticity. Thus, aluminum adjuvants have been described as difficult to manufacture in a physicochemically reproducible way, thus resulting in batch to batch variations [6,13,16]. To minimize the variations and to avoid the non-reproducibility due to use of different preparations of aluminum compounds, a specific preparation (Alhydrogel®, aluminum hydroxide, from Superfos Biosector, Vedbaek, Denmark) was chosen as a scientific standard for evaluation of new adjuvant formulations [17]. However, certain antigens do not adsorb onto Alhydrogel® due to same charge on the adjuvant and antigens [7,18]. Therefore, selection of appropriate aluminum adjuvant to give an optimal adjuvant effect is very important.

2.1. In situ precipitation of aluminum gels in presence of antigens

In situ precipitation of tetanus and diphtheria toxoids in culture medium used for growing the organisms containing anions including phosphate, sulphate and bicarbonate ions with potassium or sodium alum, is the original method developed primarily for purifying toxoids [15,19]. Vaccines

prepared by this method were referred to as alum-precipitated toxoids and were more immunogenic than the soluble formulations. Alum-precipitated vaccines were a mixture of aluminum compounds, mainly aluminum phosphate and aluminum hydroxide. This product was highly heterogenous [6] and difficult to manufacture in a consistent and reproducible manner [3,8,9,12]. In 1976, a World Health Organization report [20] described this method as a laboratory procedure which did not define the nature of the material obtained either quantitatively or qualitatively. For these reasons this product is not very common now. In situ adsorption of antigens on aluminum phosphate has also been carried out by suspending purified vaccine antigens in dibasic or tribasic sodium phosphate or phosphate buffer and precipitating with aluminum chloride [5,7]. This type of reaction can be carried out under controlled conditions and results in a consistent product.

2.2. Adsorption of antigen on pre-formed aluminum gels

Currently, the most commonly used method for preparation of aluminum adsorbed vaccines is adsorption of antigens on preformed aluminum phosphate or aluminum hydroxide gels under controlled conditions [5,15]. These preparations are usually referred to as aluminum phosphate or aluminum hydroxide adsorbed or adjuvanted vaccines. Adsorption is carried out by incubating the gel and the antigen, at optimal pH, with slow stirring for a few hours to overnight [6]. Adsorption of antigens is also carried out on freshly prepared aluminum phosphate gel [21]. Gels of aluminum phosphate and aluminum hydroxide, of clinical grade, are commercially available.

3. Factors affecting adsorption

Adsorption of antigens on aluminum salts depends heavily on electrostatic forces between adjuvant and antigen [5,7,18]. Other interactions including hydrophobic, van der Waals and hydrogen bonding contribute to the adsorption of antigens on aluminum adjuvants. However, these forces may not suffice to cause adsorption of antigen if the same charge or electrostatic repulsive force is present on antigen and adjuvant. The two most commonly used aluminum adjuvants, aluminum hydroxide and aluminum phosphate, have different points of zero charge [7,22]. At neutral pH these gels have opposite charges, wherein aluminum phosphate is negatively charged and aluminum hydroxide is positively charged. It is important to select the aluminum adjuvant carefully on the basis of the charge of the antigen at neutral pH. Other physical conditions affecting adsorption of antigens on aluminum adjuvants include pH, temperature, size of the gel particles and ionic strength of the reaction mixture [2,5–7,12,14,15,23]. The pH and ionic strength affect adsorption by altering charge on the gel and the antigens, whereas the temperature may affect the rate of interaction between the gel and the antigen. Size of gel particles affects the surface area of gel available for adsorption: small particles have more surface area than large particles. For example, amount of diphtheria toxoid adsorbed on to aluminum hydroxide gels was inversely proportional to the gel particle size [23].

Acidic pH of less than 6 has been found optimal for adsorption of several antigens on aluminum adjuvants [12]. For example, the optimal pH for adsorption of tetanus and diphtheria toxoid onto aluminum phosphate is 6.0–6.3 [5]. The adsorption of diphtheria toxoid onto aluminum phosphate is heavily influenced by pH and the presence of excess phosphate ions in the reaction mixture (Table 1 and Table 2). Aluminum phosphate and diphtheria toxoid are both negatively charged at neutral pH (see Section 4) resulting into poor adsorption. At pH 6, aluminum phosphate is positively charged, thus improving adsorption of negatively charged diphtheria toxoid. In situ adsorption of diphtheria toxoid resulted in higher adsorption than the commercial aluminum phosphate preparation (Table 1), probably due to trapping of some antigen in the gel. Adsorption of tetanus and diphtheria toxoids onto aluminum hydroxide gel (Alhydrogel®) was not sensitive to the conditions of pH and excess phosphate ions (Table 1) [4,5] because it is positively charged at pH 6 and 7.

Adsorption of bovine serum albumin on to aluminum hydroxide and lysozyme onto aluminum phosphate was inversely proportional to the ionic strength [18]. Excess anions, particularly phosphate

Table 1
Effect of pH and excess phosphate ions on the adsorption of diphtheria toxoid onto aluminum adjuvants

Adjuvant type and Concentration	Conditions of Adsorption		% Adsorption[a] Diphtheria Toxoid
	Medium	pH	
Aluminum phosphate in situ[b], 1.36 mg/ml	PBS[c]	7.2	0
		6.0–6.3	57
	Saline	7.2	13
		6.0–6.3	100
Aluminum phosphate Freshly made[d], 4 mg/ml	PBS[c]	7.2	0
		6.0–6.3	93
	Saline	7.2	52
		6.0–6.3	93
Aluminum phosphate Adju-Phos®[e], 2 mg/ml	PBS[c]	7.2	0
		6.0–6.3	46
	Saline	7.2	0
		6.0–6.3	55
Aluminum hydroxide Alhydrogel®[e], 2 mg/ml	PBS[c]	7.2	99
		6.0–6.3	100
	Saline	7.2	100
		6.0–6.3	100

[a]Diphtheria toxoid at 20 Lf/ml was adsorbed onto various gels at room temperature overnight and supernatants after centrifugation were assayed for unadsorbed antigen by a sandwich-type capture ELISA [21].
[b]In situ adsorption on aluminum phosphate was carried out by suspending the antigens in phosphate buffer (1.0 × conc., see Table 2), precipitating with aluminum chloride and adjusting pH with sodium hydroxide.
[c]PBS = Phosphate-buffered saline with 0.01 M phosphate buffer.
[d]Prepared by precipitation of trisodium phosphate and aluminum chloride, adjusting pH with sodium hydroxide, followed by addition of antigens [21].
[e]From Superfos Biosector, Vedbaek, Denmark
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Table 2
In situ adsorption of diphtheria toxoid at 20 Lf/ml onto aluminum phosphate gel (0.3 mg aluminum per ml) with varying concentration of excess phosphate ions present during precipitation

Excess Phosphate buffer conc.[a]	pH	% Adsorption
1.0 ×	6.00[b]	100
1.0 ×	6.40[b]	78
3.8 ×	6.00	60
5.0 ×	6.25	25
10.0 ×	6.75	0
10.0 ×	6.00[c]	34

[a]0.792 ml of 1.12 M phosphate buffer (a mixture of Na_2HPO_4, 12.5 g and $Na_2PO_4.H_20$, 3.3 g in 100 ml) for 100 ml of gel is 1.0 × conc. For the preparation of in situ aluminum phosphate gel, antigens in a total volume of 100 ml with 0.792 ml of 1.12 M phosphate buffer are precipitated with 2.7 ml of 10% $AlCl_3.6H_20$ and pH adjusted immediately to 6 with sodium hydroxide.
[b]pH adjusted with sodium hydroxide immediately after forming the gel.
[c]pH adjusted with hydrochloric acid immediately after forming the gel.
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ions, and impurities, such as amino acids, peptides and polysaccharides, reduce protein adsorption, probably by competing with antigen for adsorption sites [14]. Multiple-charged negative ions, especially phosphate ions, interfere with the adsorption capacity of aluminum hydroxide and these may be used for eluting adsorbed antigens from the gel [6]. Lindblad and Sparck [14] recommended avoiding phosphate-buffered saline in the reaction mixture for adsorption of antigen onto aluminum adjuvants. In general, a low ionic strength and absence of excess phosphate ions and impurities are recommended for optimal adsorption of antigens on aluminum phosphate gel [15]. The temperature of adsorption has been considered important for complete adsorption of antigen onto aluminum phosphate although most of the adsorption, up to 80–90% of diphtheria toxoid, occurs within a few min at temperatures ranging from 4° to 45°C [12].

Aluminum hydroxide (Alhydrogel®) was capable of adsorbing higher amounts of tetanus toxoid (273.4 Lf or ~820 μg per mg of gel) and diphtheria toxoid (126.6 Lf or ~380 μg per mg of gel) than aluminum phosphate (Adju-phos®) (53.5 Lf or ~161 μg tetanus toxoid/mg gel) at a pH of 6.0 [5]. Nicklas [11] described adsorption of 50–200 μg of protein/mg aluminum hydroxide gel. Lindblad and Sparck [14] found 10–20 times more adsorption of human serum albumin on aluminum hydroxide than on aluminum phosphate. Aggerbeck and Heron [24] adsorbed 400 Lf of diphtheria toxoid and 100 Lf of tetanus toxoid completely on 1 mg of aluminum as aluminum hydroxide. Therefore, aluminum hydroxide has higher adsorption capacity than aluminum phosphate, particularly for routine childhood vaccine antigens, tetanus and diphtheria toxoids. The major reason is the charge on these gel at neutral or slightly acidic pH (6.0). As discussed below (Section 4), aluminum hydroxide has strong positive charge at pH 6–7, whereas aluminum phosphate has a weak or

neutral charge at this pH range. Tetanus and diphtheria toxoids being negatively charged at pH 6–7 show strong and more adsorption to aluminum hydroxide than to aluminum phosphate.

There has been some discussion on the desorption of antigen from adjuvant after injection into the body where a physiologically neutral pH and presence of body fluids containing proteins and anions that might desorb the antigen from the gel. Earlier studies showed that freshly made preparations of aluminum phosphate adsorbed diphtheria toxoid had more antigen desorption than aged preparations when exposed to neutral pH or serum [12]. Thus, aging of aluminum- adsorbed vaccines appears to improve their immunogenicity [8].

4. Physico-chemical characteristics

Stanley Hem and coworkers have extensively studied the physico-chemical characteristics of aluminum adjuvants and their effects on the adsorption of proteins on to these adjuvants [7,18,22,25–28]. Aluminum hydroxide has been identified as poorly crystalline aluminum oxyhydroxide with a structure of the mineral boehmite. It has high surface area with a pI of 11, which favors adsorption of negatively charged proteins at neutral pH. In contrast, aluminum phosphate and alum-precipitated vaccines have been classified as amorphous aluminum hydroxyphosphate with little sulfate. Depending upon the conditions under which these gels are prepared, the molar ratio between aluminum and phosphate of amorphous aluminum hydroxyphosphate varies which results in pI values from 5 to 7. So these gels are negatively charged or without any charge at neutral pH. The amorphous nature of these compounds contributes to high surface area and high protein adsorption capacity, mainly for positively charged proteins. That is the reason for poor adsorption of negatively charged diphtheria toxoid onto aluminum phosphate at a neutral pH. But, aluminum phosphate gel with a pI close to 7 would be positively charged at pH of 6.0, leading to adsorption of diphtheria toxoid. Therefore, formulation of DTP vaccine with aluminum phosphate is usually done at a pH close to 6.0 to allow maximum adsorption of diphtheria toxoid.

The physico-chemical characteristics are very useful in optimizing the adsorption of antigens onto aluminum adjuvants and for formulation of combination vaccines. By controlling the physico-chemical characteristics of aluminum adjuvants and vaccines adsorbed onto these adjuvants, it would be easy to control the manufacturing process of these vaccines and achieve consistency in production as per good manufacturing practices.

5. Adjuvant properties

The adjuvanticity of aluminum adjuvants for human vaccines, particularly tetanus and diphtheria toxoids, was clearly established during the 1930's [29–32]. Thereafter, the use of aluminum adjuvants became common, although a few reports stated that alum-adjuvanted vaccines were not better than soluble vaccines [9]. The major advantage of using aluminum adjuvants was the more rapid development of high titered and long-lasting antibody responses after primary immunization [31,32]. There are numerous reports in humans and animals showing the superiority of aluminum adsorbed tetanus and diphtheria toxoids over soluble toxoids, particularly after the first dose [9,15,21,33–39]. However, aluminum-adsorbed vaccines did not show any advantage over soluble preparations for the booster or secondary response [21,39–43]. Recently, it was reported that soluble or calcium phosphate adsorbed toxoids were more immunogenic in humans than toxoids adsorbed onto aluminum adjuvants, when used as booster injections [43–47]. However, tetanus toxoid adsorbed onto aluminum adjuvants was more reliable than soluble toxoid when given simultaneously with an injection of tetanus antitoxin [34,48,49], because soluble toxoid and antitoxin would be cleared fast by the interaction between themselves which would reduce the effectiveness of both.

Aluminum adjuvants are universally used with diphtheria, tetanus and pertussis (DTP) vaccines, although, the adjuvant effect on whole cell pertussis component is not clear. Although serum agglutinins to *Bordetella pertussis* produced after immunization with aluminum adjuvanted pertussis vaccine were higher than those obtained after inoculation with

unadsorbed pertussis vaccine [50–54], there was no difference between unadsorbed and adjuvanted pertussis vaccine with regard to protection against disease [55]. The adjuvant effect of aluminum phosphate or aluminum hydroxide on the mouse intracerebral potency of whole cell pertussis vaccine is controversial. In a few studies, the potency of adjuvanted vaccine was higher than the non-adjuvanted pertussis vaccine [56–59] but in other studies, the adjuvant did not have any effect [9,60–62]. Aluminum compounds are routinely used with the new acellular pertussis vaccines [63–67].

Aluminum compounds have also been used with inactivated polio vaccine [68], human diploid cell strain rabies vaccine [69], hepatitis B vaccine [70] and hepatitis A vaccine [71,72]. Aluminum hydroxide adsorbed cholera vaccine provided better protection than the unadsorbed cholera vaccine [73]. Variable results were obtained with *Haemophilus influenzae* type b (Hib) conjugate vaccines when given with aluminum adjuvants. An earlier study did not show an adjuvant effect of aluminum adjuvants with one Hib polysaccharide-protein conjugate vaccine [74], but aluminum adjuvants have been used successfully with another Hib conjugate vaccine [75] as well as Hib conjugate vaccines that are given in combination with DTP vaccines [76,77]. In a recent study, Hib conjugate and Meningococcus type C conjugate vaccines were found to be immunogenic in infant baboons when given with aluminum hydroxide or another experimental adjuvant MF59 but not in the plain form [78]. The infant baboon model has been described as a reliable predictor of immunogenicity of Hib and Meningococcus type C conjugate vaccines in infants [78]. Aluminum adjuvants have also been widely used with a number of veterinary vaccines [2,6], including vaccines against avian infectious bronchitis [79], canine hepatitis [80], foot and mouth disease [81,82], Newcastle disease [83], *Bacteroides nodosus* [84], *Bordetella bronchiseptica* [85], *Pasteurella multocida* [86], *Leptospira interrogans* [87], *Cooperia punctata* [88], *Nematospiroides dubius* [89], *Nematospiroides dubius* [90], *Trichinella spiralis* [91]. Thus, aluminum adjuvants have wide applications with both human and veterinary vaccines.

5.1. Role of adsorption on adjuvanticity

The immunogenicity of antigens adsorbed onto aluminum adjuvants appears to depend on the degree of antigen adsorption and the dose of adjuvant [4,5]. Data in Table 3 demonstrate this point. The formulation which did not show any adsorption of diphtheria toxoid onto aluminum phosphate, due to presence of a ten-fold excess of phosphate, did not elicit an

Table 3
Effect of degree of adsorption of diphtheria toxoid onto aluminum adjuvants on immunogenicity in mice[a]

Adjuvant	Dose per mouse		Adsorption (%)	GM[b] anti-DT IgG (μg/ml) after	
	Adjuvant (mg)	DT (μg)		First dose	Second dose
Aluminum phosphate (in situ)	0.068	1.5	0	0.04 (0.01--0.22)	8.1 (1.4--46.8)
	0.068	1.5	90	10.97(4.60–26.3)	380.9(211.7–685.4)
	0.068	1.5	100	9.40(3.94–22.4)	361.3(169.8–769.1)
	0.068	3.0	78	2.95(0.96– 9.00)	157.8(54.4–457.6)
Aluminum phosphate (Freshly made)	0.200	3.0	100	62.60(44.9–87.4)	919.3(643.1–1314.1)
Aluminum hydroxide (Alhydrogel®)	0.033	1.5	100	49.10(19.3–125.1)	424.6(162.6–1108.6)
	0.033	3.0	100	29.20(12.9–65.7)	791.8(345.0–1817.3)
	0.100	1.5	100	45.00(17.3–116.9)	1021.2(573.2–1819.4)
None	–	1.5	–	0.03	0.3

[a]Four week old female outbred (CD-1 strain) mice were injected with diphtheria toxoid subcutaneously twice at an interval of 30 days. Mice were bled 4 weeks after the first and two weeks after the second dose. IgG antibodies to diphtheria toxoid were determined in the sera of individual mice by ELISA [117].

[b]GM = Geometric mean with 95% confidence intervals in parenthesis.

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antibody response after the first injection and only a poor response after the second dose. There has been a debate on the optimal degree of adsorption and its role in the adjuvanticity of aluminum adjuvants in humans since there is hardly any study in humans on this topic. Vaccines with less adsorption of antigens onto aluminum adjuvants, ~50% or even lower adsorption, have proved effective in the field and meet all the requirements of the National Control Authorities. Despite these controversies and uncertainty about precise mechanism of action of aluminum adjuvants, adsorption is still considered to be a very important parameter for the function of these adjuvants [5]. Thus, measuring the degree of adsorption is one of the parameters that can be controlled in the formulation process during manufacture of aluminum adsorbed vaccines to achieve consistency in production. Adsorption of 80% or more of tetanus and diphtheria toxoids onto aluminum adjuvants is recommended by the World Health Organization [92]. The United States Minimum Requirements [93] for adult tetanus and diphtheria toxoid is at least 75% adsorption of diphtheria component on the aluminum adjuvants.

5.2. Dose of aluminum adjuvants

The dose of aluminum adjuvant also affects the overall immunogenicity of vaccines [15]. A small amount of aluminum adjuvant may be required for complete adsorption of the antigen, but low doses may not provide an optimal adjuvant effect. There appears to be a need for excess free adjuvant for an optimal adjuvant effect [39,94]. In animal studies, as the amount of aluminum adjuvant was increased, the adjuvant effect increased, but only to a certain concentration after which, the adjuvant effect declined [14,15,95–98]. The reasons for this optimum concentration of adjuvant are unknown. It is speculated that a certain minimum amount of aluminum compound is necessary to form a depot at the site of injection or to optimally stimulate macrophages [5]. Excessive amounts of aluminum compounds may suppress immunity by covering the antigen completely with mineral compounds [41,99] or through toxicity to macrophages [100].

The usual dose of aluminum used for human vaccines is around 0.5 mg. The upper allowable limits of aluminum adjuvants for injection in humans is 1.25 mg aluminum as per World Health Organization regulations [101] and 0.85 to 1.25 mg aluminum as per United States Food and Drug Administration guidelines [102].

5.3. Effect of dilution of final formulation on animal immunogenicity tests

Most of the biologicals, including vaccines, are evaluated in laboratory animals for potency. Doses of vaccines chosen in animals models are usually several-fold (sometimes 100's-fold) lower than the human doses to get the response on the logarithmic part of the dose-response curve. Most common practice to perform this type of assays is to dilute the vaccine formulations in saline or phosphate-buffered saline. Several studies demonstrated lower levels of immunogenicity/potency in mice and guinea pigs using aluminum-adsorbed vaccines diluted in saline compared to those diluted in the aluminum adjuvant [98,103,104]. It is believed that dilution of adjuvanted vaccines for testing in animals may disturb the composition of the vaccine [5,98,103–112]. The effect of dilution of vaccines on the immunogenicity depended upon the antigen, adjuvant and animal species. Dilution of final formulations in saline had no effect on the immunogenicity of aluminum phosphate adsorbed tetanus toxoid in mice (Table 4) whereas in guinea pigs, higher IgG antibody levels were induced using undiluted formulations (Table 5). Undiluted aluminum phosphate adsorbed diphtheria toxoid induced higher antibody levels in mice than diluted preparations (Table 6), which differs from the results obtained using tetanus toxoid. Similar results were obtained with calcium phosphate adsorbed tetanus toxoid [104, Unpublished data] and this may be the reason for lower potency in animals of calcium phosphate adsorbed tetanus and diphtheria toxoids than the toxoids adsorbed onto aluminum adjuvants [24,44] as the World Health Organization potency test requires dilution of vaccines in saline [101]. However, as discussed earlier, these calcium phosphate adsorbed toxoids were more immunogenic in humans than the corresponding aluminum adsorbed preparations [44,46,47]. Therefore, potency tests in animals based on dilution of vaccines don't provide the 'true picture' of the

Table 4
Antibody response of mice[a] to varying doses of aluminum phosphate adsorbed tetanus toxoid injected undiluted and diluted in saline

Dose Lf (~μg)	Inoculum (μl)	Antibody levels at 4 weeks	
		TN[b] (AU/ml)	IgG[c] (EAU/ml)
0.2 (0.6)	20	0.50	3.87 (2.60–5.75)
0.2 (0.6)	500	0.60	3.81 (1.86–7.79)
0.1 (0.3)	10	0.25	2.11 (1.33–3.37)
0.1 (0.3)	250	0.38	2.05 (1.34–3.15)
0.05 (0.15)	5	0.12	0.46 (0.06–3.67)
0.05 (0.15)	125	0.16	1.45 (0.88–2.37)

[a]Four week old female outbred (CD-1 strain) mice were injected subcutaneously and bled at 4 weeks.
[b]TN = Toxin neutralizing antibodies (tetanus antitoxin) were determined in Antitoxin Units per ml (AU/ml) in the pooled sera [21].
[c]IgG antibodies to tetanus toxin were determined in the sera of individual mice by ELISA and expressed in ELISA Antitoxin Units/ml (EAU/ml) [21]. Results are shown as geometric mean with 95% confidence intervals in parenthesis.

immunogenicity of the final formulation. Hence, it is recommended that for animal immunogenicity studies of adjuvanted vaccines, the formulation intended for human use should be injected undiluted or with a minimum dilution, if necessary.

5.4. Comparative adjuvanticity of aluminum compounds

Aluminum hydroxide has been found to be a more potent adjuvant than aluminum phosphate [113,114].

Table 6
Antibody response of mice[a] to varying doses of aluminum phosphate adsorbed diphtheria toxoid injected undiluted and diluted in saline

Dose Lf (~μg)	Inoculum (μl)	Antibody levels at 4 weeks IgG[b] (μg/ml)
0.2 (0.5)	10	3.30 (0.83–13.2)
0.2 (0.5)	500	0.29 (0.02–4.75)
0.1 (0.25)	5	1.29 (0.05–33.7)
0.1 (0.25)	250	0.11 (0.01–1.04)

[a]Four week old female outbred (CD-1 strain) mice were injected subcutaneously and bled at 4 weeks.
[b]IgG antibodies to diphtheria toxoid were determined in the sera of individual mice by ELISA and expressed in μg/ml [117]. Results are shown as geometric mean with 95% confidence intervals in parenthesis.

This may be due to its overall higher adsorption capacity and better adsorption properties of certain antigens at neutral pH (Table 1). Aluminum hydroxide adjuvanted antigens induced antibody responses that are comparable to Freund's Complete Adjuvant (FCA) [115,116]. For example, diphtheria toxoid adsorbed onto aluminum phosphate under optimal conditions induced antibody levels in rabbits similar to those elicited by the toxoid given with FCA [117]. Aluminum hydroxide is a good adjuvant for weak immunogens in mice but saponin and FCA are more potent adjuvants for more strong immunogens [15,118]. Aluminum compounds are also very potent adjuvants for tetanus and diphtheria toxoids in guinea pigs and mice, particularly in outbred CD-1 mice (Tables 3–6). The antibody responses in mice

Table 5
Antibody response of guinea pigs[a] to varying doses of aluminum phosphate adsorbed tetanus toxoid injected undiluted and diluted in saline

Dose Lf (~μg)	Inoculum (μl)	Antibody levels at		
		4 weeks IgG[b] (EAU/ml)	6 weeks	
			TN[c] (AU/ml)	IgG[b] (EAU/ml)
0.2 (0.6)	20	2.37 (1.63–3.45)	0.50	1.57 (1.17–2.12)
0.2 (0.6)	500	1.79 (0.84–3.84)	0.40	1.13 (0.71–1.79)
0.1 (0.3)	10	1.44 (0.66–3.16)	0.28	1.17 (0.59–2.31)
0.1 (0.3)	250	1.25 (0.95–1.64)	0.25	0.69 (0.53–0.90)
0.05 (0.15)	5	0.58 (0.27–1.26)	0.09	0.48 (0.22–1.06)
0.05 (0.15)	125	0.24 (0.10–0.55)	< 0.09	0.16 (0.05–0.48)

[a]Female outbred (Hartley strain) guinea pigs, 450–550 g were injected subcutaneously and bled at 4 weeks and 6 weeks.
[b]IgG antibodies to tetanus toxin were determined in the sera of individual guinea pigs by ELISA and expressed in ELISA Antitoxin Units/ml (EAU/ml) [21]. Results are shown as geometric mean with 95% confidence intervals in parenthesis.
[c]TN = Toxin neutralizing antibodies (tetanus antitoxin) were determined in Antitoxin Units per ml (AU/ml) in the pooled sera [21].

and guinea pigs after a single dose of aluminum phosphate adsorbed tetanus toxoid are routinely very high and persisted at high levels for up to a year [5,119]. Single injection of small doses (0.05 Lf or ~0.15 μg) of aluminum phosphate adsorbed tetanus toxoid elicits protective levels of antibodies in mice and guinea pigs (Table 4 and Table 5). In contrast, single injections of tetanus and diphtheria toxoids adsorbed onto aluminum adjuvants don't elicit such high antibodies in humans. Thus, animal models seem not to provide true adjuvanticity of aluminum adjuvants.

6. Mechanism of action

The mechanism of action of aluminum adjuvants is complex and not yet fully understood. It likely involves various mechanisms including the formation of depot, increasing targeting of antigens to antigen presenting cells and non-specific activation of immune system. All of these mechanisms are discussed below.

6.1. Depot formation

Depot formation by aluminum adjuvants is considered to be one of the important mechanisms of action. The depot concept has generated numerous discussions. Is it a short term depot, to which macrophages are attracted [2,132], or a long term depot from which antigen is released over a protracted time? There are also questions regarding site of the depot. Is it formed at the site of injection or in the draining lymph nodes? These questions remain unanswered with evidence to support or contradict theories.

Glenny et al. [120] proposed that aluminum adjuvants act by depot formation at the site of injection, allowing for a slow release of antigen and thus prolonging the time for interaction between antigen and antigen-presenting cells and lymphocytes. The local depot mechanism was challenged when Holt [8] described that antibody formation continued even after removal of adjuvant–antigen depot from the site of injection. In a recent study, ~90% of radio-labeled aluminum phosphate adsorbed tetanus toxoid disappeared from the site of injection within 24 h of subcutaneous injection,

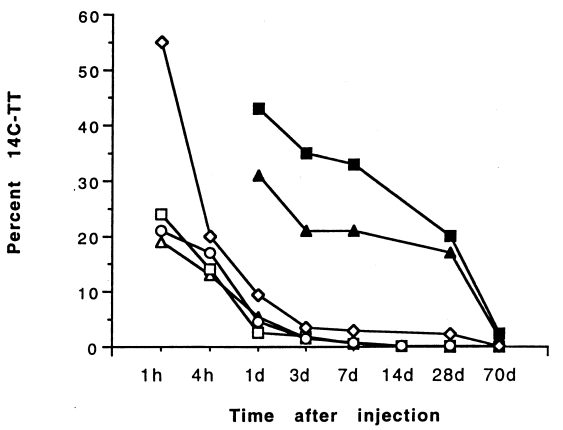

Fig. 1. Radioactivity at the site of injection in mice inoculated subcutaneously with 5 Lf (~15 μg) of soluble tetanus toxoid (○), aluminum phosphate adsorbed tetanus toxoid (◊), large microspheres containing tetanus toxoid, size ~50 μm (■), small microspheres containing tetanus toxoid, size ~5 μm (▲), mixture of large blank microspheres, size ~50 μm and tetanus toxoid (□) and mixture of small blank microspheres, size ~5 μm and tetanus toxoid (△). (Reproduced from reference [121] with permission from Elsevier Sciences).

whereas tetanus toxoid encapsulated within biodegradable polymer microspheres stayed at the site of injection for prolonged periods [121] (Fig. 1). However, amount of aluminum phosphate adsorbed tetanus toxoid at the site of injection was higher than the soluble tetanus toxoid for four weeks (Fig. 1), thus demonstrating some localized depot formation. White et al. [122] showed that antibody producing cells in the regional, popliteal, lymph nodes of rabbits injected with 150 Lf of soluble diphtheria toxoid completely disappeared in 3 weeks, whereas rabbits injected with 10 Lf of aluminum phosphate precipitated toxoid had antibody producing cells in the nodes at 3–4 weeks, suggesting a depot in the draining lymph nodes.

The most direct evidence for a local depot effect comes from experiments in which local granulomas, formed after injection of aluminum adsorbed vaccines, were able to induce immune responses when excised from the site of injection seven weeks later, macerated and injected into other animals [123]. Remarkably, the antigen in the granuloma was apparently not available to the animals for a secondary response, because minute doses of antigen injected adjacent to the granuloma produced a secondary antibody response [124]. White [124] post-

ulated that antigen at this time is unable to penetrate the fibrous tissue surrounding the granuloma and antibody may react with antigen to form an antigen–antibody precipitate within the fibers of the peripheral zone of the granuloma thus preventing the diffusion of antigen from granuloma and sequestering it from antigen presenting cells.

6.2. Targeting to antigen presenting cells

Adjuvanticity of aluminum gels may be related to their ability to convert soluble antigens to particulate forms, which are more readily phagocytosed. The particle size of commercially available aluminum adjuvant gels is less than 10 μm, the average size for aluminum hydroxide, Alhydrogel® was 3.07 μm and for aluminum phosphate, Adju-phos® was 4.26 μm [5]. It has been shown that poly lactide glycolide (PLGA) microspheres less than 10 μm are taken up by antigen presenting cells and provide strong adjuvant effects [125]. Antigen adsorbed onto aluminum hydroxide is more readily taken up by human monocytes than free antigen, and the human monocytes exposed to aluminum hydroxide secrete IL-1 [126]. Adjuvanticity of aluminum gel particles via targeting to antigen presenting cells, emphasizes the importance of degree of adsorption of antigens onto gels.

6.3. Activation of immune system

An increased antibody response to a soluble antigen has been observed when aluminum adjuvant was injected at a different site, suggesting a systemic stimulatory effect on immuno competent cells, possibly by release of inflammatory cytokines [127]. However, these results have not be confirmed. Comparison of the immune responses of mice to diphtheria toxoid and aluminum hydroxide injected at different sites versus soluble diphtheria toxoid alone [5] demonstrated that both elicited very low and similar antibody levels, even after two doses. However, aluminum compounds can induce eosinophilia [128] and activate complement [129] which may lead to a local inflammatory response, thus enhancing the antibody response.

The aluminum adjuvants augment mainly humoral immunity, particularly IgG1 and IgE antibody responses, through IL-4 [130] by activating Th2 type cells [131,132]. Aluminum adjuvants are not efficient in raising cell-mediated immune responses [16,132,133]. The induction of delayed type hypersensitivity by aluminum adjuvants in mice and guinea pigs has not been clearly demonstrated [15,134]. Cooper [131] described aluminum adjuvants as good stimulants for Th2 type cell mediated immune response, especially eosinophils. This type of response is similar to that elicited by some helminth parasites [6] and this property makes aluminum adjuvants a good candidate for anti-parasite vaccines. In a special mouse hybrid model of schistosomiasis, Horowitz et al. [135] demonstrated protection in animals injected with sonicated parasite antigen and aluminum hydroxide. In contrast, antigen injected with FCA elicited lower IgE antibodies and lower levels of protection.

7. Limitations

Aluminum adjuvants have an extensive record of safety. Billions of doses of aluminum adsorbed vaccines, particularly DTP with and without inactivated polio vaccine, have been used to inoculate children and infants. Occasionally, these vaccines have been associated with severe local reactions such as erythema [9,42], subcutaneous nodules [136], contact hypersensitivity [137] and granulomatous inflammation [122,138,139]. But in certain instances, aluminum-adsorbed DTP vaccine produced less reactions than unadsorbed vaccine [140,141] due to adsorption and subsequently slow release of reactogenic materials from the adjuvant.

In animals, as well as in humans, aluminum adjuvants increase the levels of antigen specific and total IgE antibodies [10,21,45,142–147] and may promote IgE mediated allergic reactions. In a recent study, Aggerbeck et al. [47] reported similar levels of IgE antibodies after boosters with soluble, aluminum hydroxide or calcium phosphate adsorbed tetanus and diphtheria toxoids. This was attributed to priming with aluminum adsorbed vaccines during primary immunization 20 years back [47]. Though, aluminum adjuvants have been used for many years for hyposensitization of allergic patients with satis-

factory results [3], use of such preparations has declined in recent years as it became clear that aluminum adjuvants elicit Th2 type response and stimulate the production of IgE antibodies.

Studies on booster injections, up to ten years after the last vaccination, aluminum-adsorbed vaccines induced more local reactions such as redness, swelling and itching in children who had aluminum adsorbed vaccines for primary immunization than those who had unadsorbed vaccines for primary immunization [148–150]. Antigen-specific IgE antibody levels following booster injections were also higher in the group which had aluminum adsorbed vaccines for primary immunization [148]. However, there were no differences in the frequency of local reactions and IgG antibody responses to booster doses of soluble or aluminum phosphate adsorbed diphtheria-tetanus toxoid in children who had primary immunization 10 years earlier with aluminum adsorbed vaccines [43]. Nevertheless, the children boosted with aluminum phosphate adsorbed vaccine developed higher levels of tetanus specific IgE than those boosted with unadsorbed vaccine [45] and higher levels of antigen-specific IgE correlated with local reactions [45,149]. Taken together these studies suggest that children who had primary immunization with aluminum-adsorbed vaccines are more likely to develop antigen specific IgE and a higher frequency of local reactions on booster injection with soluble or aluminum adsorbed vaccines than children who had primary immunization with unadsorbed vaccines. Based on these observations, Mark et al. [45] suggested a need to re-evaluate aluminum compounds as vaccine adjuvants. There have been concerns, particularly in patients with impaired renal function, about systemic accumulation of aluminum which has been associated with nervous system disorders and bone diseases [151,152]. However, aluminum intake from vaccines is minor compared to that of diet and medications, such as antacids.

Other limitations of aluminum adjuvants include their ineffectiveness when used with certain antigens [133] and the induction of mainly humoral immunity and Th2 type responses [132,153]. Aluminum compounds did not exhibit an adjuvant effect when used with typhoid vaccine [154], influenza haemagglutinin antigen [155] and Hib capsular polysaccharide-tetanus toxoid conjugate [74]. However, aluminum hydroxide has been successfully used with another conjugate vaccine composed of Hib capsular polysaccharide linked to outer membrane proteins of *Neisseria meningitidis*. The adsorbed preparations produced fewer local and systemic reactions than the soluble preparation, probably due to slow release of vaccine [75]. The inability of aluminum adjuvants to elicit cell mediated immune responses, particularly cytotoxic T-cell responses [132], may be a significant limitation for vaccines against intracellular parasites and some viruses, such as human immunodeficiency virus.

Aluminum is not 'biodegradable' [156]. Aluminum adjuvants have been found at the site of subcutaneous injection in mice and guinea pigs for up to one year [119,121]. In contrast, PLGA microspheres, which also form a long term depot at the site of injection, degrade and disappear from the site of injection between 3 to 12 months, depending upon the characteristics of polymer from which the microspheres are made [119,121]. Though biodegradation of aluminum adjuvants may not have a clinical significance, PLGA microspheres with similar or better adjuvanticity may be preferred over aluminum adjuvants. In recent years, a lot of progress has been made with the PLGA microsphere-containing vaccines (discussed in a separate review of this issue), but there are still a large number of unresolved scientific and regulatory issues with such vaccines.

Aluminum adjuvants cannot be frozen or easily lyophilized [16,157] as both of these processes cause the collapse of the gel resulting in gross aggregation and precipitation. Although tetanus toxoid with collapsed gel precipitates was found to be immunogenic [37], such a vaccine is not clinically acceptable. Successful lyophilization of aluminum adjuvants was reported [158] but lyophilized vaccines containing aluminum adjuvants are not available commercially. Use of a lyophilized DTP vaccine with acellular pertussis components adsorbed onto aluminum adjuvants and stabilized with Haemocoel and sucrose has also been described [159]. Though Haemocoel has been used for lyophilization of International Standards and Reference Preparations of aluminum adsorbed vaccines, it has not been used in human vaccines. The progress in this field is slow as there does not seem to be a need for a lyophilized aluminum adsorbed vaccine in the developed coun-

tries. With the improvement of cold chain, such a vaccine is also not a priority in developing countries.

8. Alternatives to aluminum adjuvants

There has been a search for other adjuvants for human vaccines for many years [2–4,13–16,21,78,115,131–134,157]. Several adjuvants have been or are being evaluated, but none except calcium phosphate has been used routinely with vaccines for humans [3–5,10,21,24,44,46,47,101,147,151,160–165]. Calcium phosphate has been successfully used in France with routine childhood and adult vaccines for many years and has been found to be safe and efficacious in various field trials. Calcium phosphate is a normal constituent of the body and is well tolerated and readily resorbed. Unlike aluminum adjuvants, calcium phosphate does not enhance IgE production in humans and animals. Use of calcium phosphate adsorbed vaccines was discontinued in France as these vaccines had problems in passing the potency for tetanus and diphtheria components as per the World Health Organization and European Pharmacoepia regulations though these vaccines were highly immunogenic in humans [44,46,47]. As discussed earlier, this was due to design of these assays using diluted vaccines (see Section 5.3).

9. Combination of aluminum compounds with other adjuvants

Aluminum compounds have also been used in combination with other adjuvants. However, these combinations are not described in detail in this review. Historically, aluminum adjuvants have been used with DTP vaccines which contain two other adjuvant active components from pertussis organisms, specifically lipopolysaccharide and pertussis toxin [3]. The adjuvant effect of pertussis vaccine is clearly evident on tetanus toxoid by increase in its potency in mice [101]. Aluminum compounds have also been used with liposomes and monophosphoryl lipid A [16], γ inulin [131] and QS21 [166]. Recently, the use of aluminum adsorbed tetanus toxoid with the toxoid also encapsulated in PLGA polymer microspheres has been suggested for the development of a single dose tetanus vaccine [167,168]. It is believed that the aluminum adsorbed tetanus toxoid initiates the immune response and microencapsulated toxoid boosts and maintains the antibodies at high levels by continuous release of antigen. Thus, aluminum adjuvants are compatible with other adjuvants and delivery systems for vaccines.

10. Conclusions

It is likely that aluminum compounds will continue to be used with human vaccines for many years due to their excellent safety and adjuvanticity records and compatibility with a variety of antigens. Aluminum adjuvants formulated under optimal conditions elicit rapid, high and long lasting antibody responses to a number of antigens after primary immunization. Limitations of aluminum adjuvants include local reactions, augmentation of IgE antibody responses, ineffectiveness for some antigens and inability to elicit cell-mediated immune responses especially cytotoxic T-cell responses. These limitations support the need for continuous research with adjuvants that are more potent and can selectively modulate the immune response to the desired type.

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