



Calcium phosphate: a substitute for aluminum adjuvants?

Jean-Daniel Masson, Michel Thibaudon, Laurent Bélec & Guillemette Crépeaux

To cite this article: Jean-Daniel Masson, Michel Thibaudon, Laurent Bélec & Guillemette Crépeaux (2016): Calcium phosphate: a substitute for aluminum adjuvants?, Expert Review of Vaccines, DOI: [10.1080/14760584.2017.1244484](https://doi.org/10.1080/14760584.2017.1244484)

To link to this article: <http://dx.doi.org/10.1080/14760584.2017.1244484>



Accepted author version posted online: 03 Oct 2016.
Published online: 24 Oct 2016.



Submit your article to this journal [↗](#)



Article views: 15



View related articles [↗](#)



View Crossmark data [↗](#)

REVIEW

Calcium phosphate: a substitute for aluminum adjuvants?

Jean-Daniel Masson^a, Michel Thibaudon^b, Laurent Bélec^c and Guillemette Crépeaux^{d,e}

^aAssociation E3M (Entraide aux Malades de Myofasciite à Macrophages), Monprimblanc, France; ^bPharmacien « Service des Allergènes », de l'Institut Pasteur, Paris, France; ^cLaboratoire de Microbiologie, hôpital Européen Georges Pompidou, Assistance Publique-Hôpitaux de Paris, & Faculté de Médecine Paris Descartes, Université Paris Descartes, Sorbonne Paris Cité, Paris, France; ^dÉcole nationale vétérinaire d'Alfort, Maisons-Alfort, France; ^eInserm U955 E10, Université Paris Est Créteil, Créteil, France

ABSTRACT

Introduction: Calcium phosphate was used as an adjuvant in France in diphtheria, tetanus, pertussis and poliomyelitis vaccines. It was later completely substituted by alum salts in the late 80's, but it still remains as an approved adjuvant for the World Health Organization for human vaccination.

Area covered: Thus, calcium phosphate is now considered as one of the substances that could replace alum salts in vaccines. The aim of this paper is to draw a review of existing data on calcium phosphate as an adjuvant in order to bring out the strengths and weaknesses for its use on a large scale.

Expert commentary: Calcium phosphate is a compound naturally present in the organism, safe and already used in human vaccination. Beyond comparisons with the other adjuvants, calcium phosphate represents a good candidate to replace or to complete alum salts as a vaccine adjuvant.

ARTICLE HISTORY

Received 12 May 2016
Accepted 30 September 2016
Published online 25 October 2016

KEYWORDS

Vaccine; adjuvant; calcium phosphate; safety; immune response; aluminum salts

1. Introduction

Adjuvants used in vaccines can be defined as substances added to antigens to enhance immunity response to these antigens [1,2]. First pastorian vaccines, such as rabies vaccine, were developed at the end of the nineteenth century without adjuvants because they were designed from whole viruses or bacteria. The notion of adjuvant was developed at the beginning of the twentieth century when traditional technique failed to produce an efficient immunity response in horses with diphtheria and tetanus purified toxoids [3,4].

(Toxoid = toxin with intact immunogenic properties, but whose toxicity has been destroyed by heat or formaldehyde for example.)

Concomitantly, alum salts (aluminum hydroxide $\text{Al}(\text{OH})_3$ and aluminum phosphate AlPO_4) have shown their effectiveness to produce a quality immune response with the same toxoids as in horses. Indeed, the injection of alum potassium ($\text{KAl}(\text{SO}_4)_2$) with diphtheria toxoid produced higher plasma concentration of antitoxin than the same vaccine without alum [5].

The substantially equivalent result in humans explains that aluminum salts are now the most frequently used adjuvants although other substances such as oil in water emulsion, saponin, or polysaccharides have been developed too [4].

Adjuvants such as aluminum salts aim to improve the vaccine antibody response in order to make them more effective [3]. However, one should recall controversies on these adjuvants, notably the one dealing with hepatitis B and human papillomavirus vaccine, causing autoimmune or neurological diseases [6]. Among unusual reactions to vaccines

containing aluminum hydroxide, macrophagic myofasciitis is an inflammatory lesion described in 1998 [7] and recognized as a 'distinctive histopathological entity that may be caused by intramuscular injection of Al-containing vaccines' [8,9]. Diseases attributed to vaccines, quoted in this article, have in common the presence of neurological disorders. The neurotoxicity of aluminum is now well recognized, and the possible relationship between aluminum and chronic neurological diseases such as Alzheimer's disease has been the key issue of studies for many years [4,10,11].

The total safety of aluminum adjuvant for human use is still debated [12]. Many studies demonstrate the link between vaccinal aluminum and different diseases, but the French National Academy of Medicine has not admitted this relationship yet [4,13].

Nevertheless, the question of vaccine safety is of significant concern and associations of patients advocate, as a precaution measure, the substitution of Al-adjuvant by calcium phosphate, which is another adjuvant already used in human vaccination.

Calcium phosphate was initially developed by the Pasteur Institute in hydroxyapatite form ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) although the patent describes it as the tricalcium phosphate form ($\text{Ca}_3(\text{PO}_4)_2$) (see Section 2.1.) [14]. This adjuvant was used in diphtheria, tetanus, pertussis, and poliomyelitis vaccines [15–17]. The preparation method of this compound is detailed in Section 2.1. Although calcium phosphate was later substituted by alum salts in the late 1980s, when the industrial sectors of Pasteur Institute and Merieux Institute merged, it still remains as an approved adjuvant for the World Health Organization (WHO) for human vaccination.

Thus, calcium phosphate is now considered as one of the substances that can replace the alum salts in vaccines [18,19]. The nonspecific use of calcium phosphate with any antigen has already been proposed but was never fully researched due to the success of the aluminum adjuvants [20].

However, since 1984, Pasteur Institute has marketed delayed-effect allergens adsorbed on calcium phosphate in order to highlight the delay effect of calcium phosphate and its immunogenic effect. As the use of allergens in specific desensitization required an increasing number of injections and since the only delayed-effect allergens were allergens on aluminum hydroxides (alum-pyridine or aluminum hydroxide *in situ*), Pasteur Institute then moved towards the only alternative to aluminum hydroxide: calcium phosphate [21].

Recent demands of associations of patients to use again calcium phosphate instead of vaccinal aluminum have revived studies on this molecule, but very few data are available in the scientific literature. Opinions differ about efficiency and innocuity of calcium phosphate adjuvants. In this context, we intend to draw a review of existing data on calcium phosphate as an adjuvant in order to bring out the strengths and weaknesses for its use on a large scale.

2. Adsorption capacity and adjuvanticity

In the lights of the currently available scientific literature, we should clearly define the concepts of adsorption capacity and antigenicity, which sometimes seem confused. We will therefore consider that adsorption capacity is the adjuvant capacity to adsorb a certain amount of antigen per unit of volume. Antigenicity is the capacity of an adjuvant to induce an adequate immune response to vaccination in terms of antibody blood concentration or titre. In other words, its capacity to present an antigen in an efficient manner to induce the optimal production of antibodies by the organism according to the initial antigen dose injected. This point was also fundamental in the choice of calcium phosphate as adjuvant adsorbed with allergens for specific desensitization [21].

2.1. Preparation and quality of calcium phosphate

Two preparation methods are possible to obtain vaccinal calcium phosphate. The first is the *in situ* co-precipitation with disodium hydrogen phosphate, calcium chloride, and antigen (gel form). The second is the direct adsorption onto a preformed gel (suspension form) [15,20,22–24]. The calcium phosphate gel form is prepared by contacting an antigen with an aqueous gel obtained through a reaction between an aqueous solution of dibasic sodium phosphate (Na_2HPO_4) and an aqueous solution of calcium chloride (CaCl_2). The calcium chloride solution is poured into the phosphate solution as rapidly as possible (within 3 min), in a proportion substantially equal to 1 mol of Na_2HPO_4 for 1 mol of CaCl_2 , stirring constantly. The mixture thus obtained is stirred on and on while its pH is adjusted to a value very near 7 with sodium hydroxide, and the gel formed is subsequently decanted and washed. The gel is composed of calcium and phosphate ions in proportions such that the ratio Ca/P is 1.62–

Table 1. Presentation of calcium phosphate compositions (formula, name, and Ca/P ratio).

Ca/P ratio	Formula	Name
0.5	$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$	Monocalcium phosphate monohydrate
1.0	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	Hydrated calcium phosphate/brushite
1.0	CaHPO_4	Anhydrous calcium phosphate/monetite
1.33	$\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$	Octacalcium phosphate
1.5	$\text{Ca}_3(\text{PO}_4)_2$	Tricalcium phosphate/whitlockite
1.5	$\text{Ca}_9(\text{HPO}_4)(\text{PO}_4)_5(\text{OH})$	Calcium-deficient hydroxyapatite
1.67	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	Hydroxyapatite
2.0	$\text{CaO} \cdot \text{Ca}_3(\text{PO}_4)_2$	Tetracalcium phosphate/hilgenstockite

1.85 and exhibits a settling rate of 1–20 mm in 10 min at 20°C when containing 0.07 atoms Ca per liter [14,17,22–24].

Calcium phosphate can be found in many forms presenting different Ca/P ratios (Table 1). The theoretical molecular composition of calcium phosphate used in vaccination is $\text{Ca}_3(\text{PO}_4)_2$ [25], but in the filed patent, the ratio Ca/P is from 1.62 to 1.85, which approximates the ratio of hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) [14]. Although the ratio Ca/P is not sufficient to define a compound, it seems that the calcium phosphate adjuvant is not chemical tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) as the name implies but hydroxyapatite (hereafter referred to as calcium phosphate) under different forms with different Ca/P ratios including the calcium-deficient (non-stoichiometric) hydroxyapatite ($\text{Ca}_9(\text{HPO}_4)(\text{PO}_4)_5(\text{OH})$) that have the same ratio Ca/P as tricalcium phosphate [26]. The presence of a hydroxyl group is important as compounds containing metallic hydroxyls exhibit a pH-dependent surface charge. In addition, adsorption of phosphorylated antigens by ligand exchange with surface hydroxyls may also occur, thus impacting on the adsorption capacity of the adjuvant [26–28].

The quality of the calcium phosphate gel depends on the concentration of the reactants and the rate at which the reactants are mixed. The best adjuvant quality was obtained in equimolar solution with a quick mixing (shorter than 3 min) [17]. Indeed, in the patent, the author observed that the very rapid addition of the calcium salt leads to a calcium phosphate compound that differs from brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) and other forms [14]. Slow mixing of the reactants (longer than 3 min) results in a gel with a calcium-to-phosphorus ratio of 1.35–1.55, whereas quick mixing of the reactants (10 s) gives a calcium-to-phosphorus ratio of 1.83 (Table 2) [14,17].

The mixing rate of reactants also affects the physical characteristics and adsorption of antigens onto the gel. Calcium phosphate gel prepared by rapid pouring of calcium chloride into disodium hydrogen phosphate and mixing within 10 s results in a gel with a lower pH and that sediments more slowly than the calcium phosphate gel made by slow mixing

Table 2. Effect of reactant mixing time (equal volumes of 0.07 M disodium hydrogen phosphate and 0.07 M calcium chloride) on the chemical composition of calcium phosphate gel.

Reaction time ^a	Concentration (mg/mL) in				
	Reactants		Gel		Ca/P ratio
	Ca	P	Ca	P	
10 s	1.41	1.07	1.38	0.76	1.83
10 min	1.47	1.15	1.40	1.03	1.35
20 min	1.43	1.12	1.39	0.92	1.51
30 min	1.44	1.07	1.39	0.90	1.55

^aTime taken to pour calcium chloride into disodium hydrogen phosphate.

Table 3. Effect of reactant mixing time (equal volumes of 0.07 M disodium hydrogen phosphate and 0.07 M calcium chloride) on the physical characteristics of calcium phosphate gel.

Reaction time ^a	pH of gel	Height ^b (mm) of clear solution in					
		mL/L of NaOH to bring the pH to 6.85	5 min	10 min	20 min	75 min	17 h
10 s	5.7	14.8	2.5	3.8	6.3	21.2	85.0
10 min	6.5	1.2	48.0	82.0	92.0	100.0	105.0
20 min	6.0	7.0	48.0	83.0	91.0	95.0	105.0
30 min	6.1	5.2	57.0	89.0	95.0	105.0	109.0

^aTime taken to pour calcium chloride into disodium hydrogen phosphate.

^bHeight of clear liquid at top of gel in 125-mm-long tube containing 50 mL of gel, held at 20°C.

of reactants (≥ 10 min) (Table 3). Calcium phosphate adjuvant precipitated by rapid mixing adsorbed 100% of diphtheria toxoid, while the adjuvant produced by slow mixing only adsorbed 58% of the same dose of diphtheria toxoid [14,17,26]. Some authors suggested a method of preparation with established conditions to produce calcium phosphate gel with constant properties [20].

2.2. Adsorption capacity

The calcium phosphate adsorption capacity is dependent on many factors and involves several phenomena such as hydrogen bonds or electrostatic attractions [26,29]. pH plays a major role in the determination of this capacity although it should be close to a physiological value for human vaccination [17]. The point of zero charge (pH at which the surface charge of a molecule is nil) is 5.5 for calcium phosphate, 5 for aluminum phosphate, and 11.1 for aluminum hydroxide. The net surface charge or zeta potential is positive when the pH is below the point of zero charge or isoelectric point. Consequently, at physiological pH, commercial calcium phosphate and aluminum phosphate adjuvants exhibit a negative zeta potential, but aluminum hydroxide exhibits a positive zeta potential [26,30,31]. An isoelectric point also characterizes antigens. As a general guideline, for many antigens, adsorption is best accomplished in the pH interval between the isoelectric point of the antigen and the point of zero charge of the adjuvant [32]. The pH-dependent zeta potential suggests that calcium phosphate adjuvant can adsorb positively charged antigens at physiological pH by electrostatic attraction. The negative zeta potential of calcium phosphate explains the effective electrostatic adsorption of lysozyme. Indeed, lysozyme has an isoelectric point of 11 and has a positive surface charge at physiological pH [26]. In the same way, this explains why aluminum hydroxide is more efficient to adsorb diphtheria and tetanus toxoid having a respective isoelectric point at 4.1 and 5 [33,34].

However, zeta potential of calcium phosphate cannot explain the good adsorption of casein, which is also negatively charged at pH 7.4. Another bonding form between calcium phosphate and the antigen is possible in addition to electrostatic attractions. The hydroxyl group in commercial calcium phosphate adjuvant makes it a candidate to strongly adsorb phosphorylated antigens as the adsorption of phosphate anion by hydroxylated mineral surfaces occurs by ligand

exchange or hydrophobic attractive forces [28]. Previous studies on aluminum hydroxide adjuvant have demonstrated that phosphorylated proteins and antigens are adsorbed by ligand exchange too [35,36]. Adsorption capacity is greatly impacted by the presence of hydroxyl group on adjuvants. Indeed, it enables the adsorption of antigen with the same surface charge as the adjuvant. Moreover, this adsorption is strong, and it is not affected by pH variation [26].

The last factor influencing the adsorption capacity is the specific surface area. This parameter takes into account the shape and the size of adjuvant particles in order to determine the real surface in contrast with the apparent surface. Calcium phosphate, as aluminum hydroxide, can be found in particle form. But contrary to aluminum hydroxide, calcium phosphate, which is a main component of bone or teeth, is a biodegradable and biocompatible material. In fact, calcium phosphate has been shown to be degraded by macrophages [37]. As the particle size decreases, the specific surface area increases. For example, two tricalcium phosphate powders used as cement have been characterized. The first one has a median particle size of 10.88 μm and a specific surface area of 0.54 m^2/g , while the second one has a median particle size of 2.22 μm and a specific surface area of 2.73 m^2/g [38]. Nanoparticles are therefore the form that presents the highest specific surface area due to its very small size. Since the adsorption capacity increases like the specific surface area, hydroxyapatite nanoparticles are the calcium phosphate form with the best adsorption capacity [39].

Due to its zeta potential, the presence of a hydroxyl group, and its specific surface area, calcium phosphate presents an adsorption capacity equal or higher than aluminum hydroxide for ovalbumin and various fractions of snake venom [40,41]. To conclude, calcium phosphate can have a good adsorption capacity of positively charged and phosphorylated antigens, and it is a biodegradable and biocompatible adjuvant even at a nanoscale.

2.3. Antigenicity

The calcium phosphates antigenicity is a topic raising many questions, and it must be highlighted that properties of calcium phosphate required for the antigenicity have not been fully characterized yet. Previous studies showed diverging and random results depending on the biological model, the used antigen, and the form (suspension or gel; see Section 2.1.) of the preparation. It seems that the adjuvant adsorption capacity does not necessarily determine its antigenicity. Indeed, for a comparable adsorption rate in antigen of snake venom, calcium phosphate gel presents a greater antigenicity than aluminum hydroxide in mice, so antigenicity does not correlate only with adsorption capacity [41]. Suspension or gel form of calcium phosphate also would have a different antigenicity, and gel form seems to have a higher antigenicity [41,42]. This antigenicity difference could come from the different properties of the compounds. Indeed, it seems likely that gel type and suspension type are not caught in the same way by phagocytes which are the source of immunogenicity [42]. Moreover, this different way taken by the suspension form could cause troubles because if calcium phosphate suspension

replaces aluminum hydroxide in vaccine formulations at the same concentration, then the potency test might show that some lots of vaccine would fail to pass the test because of small lot-to-lot variations [33].

Recent studies have shown that the physicochemical properties of particles such as the size, shape, surface charge, and surface area are important parameters that influence the adsorption capacity, antigenicity, and innocuity (for advanced information, see [43]) [26,38]. Generally, particulates with a comparable size to pathogens such as virus are easily recognized and destroyed by dendritic cells or macrophages [44]. Small particles (20–200 nm) are preferentially caught via endocytosis by dendritic cells, and larger particles (<5 µm) are phagocytosed by macrophages, leading to efficient coadministered antigen presentation and subsequent adaptive immune response [45]. Comparison of six sizes of calcium phosphate particles from 40 nm to 5 µm in mice highlighted that particles of size from 100 to 400 nm present the greater antigenicity. However, we should be careful with the trials of size because it seems that an optimal size range exists for each particulate adjuvant [37]. Commercial calcium phosphate presents a mean particle size distribution of 9.2 µm to 905 nm [46]. Antigenicity is also impacted by the vaccination schedule and booster injection (an extra amount of vaccine that is later injected to maintain an adequate level of antibody). Indeed, in humans, if antibody production to diphtheria and tetanus toxoids measured after the first injection is more important with aluminum hydroxide than with tricalcium phosphate, the final antibody production measured after the booster is more important with tricalcium phosphate [24,47].

As suggested by their successful uses in the past, the antigenicity of calcium phosphate-based vaccines may be compatible with human vaccination. The use of allergens adsorbed on calcium phosphate during desensitization treatment showed excellent immunogenicity [48]. In humans, the calcium phosphate antigenicity calculated by serum antibody concentration is important for diphtheria, tetanus, poliomyelitis, pertussis vaccines, and five associations of these various vaccines. Moreover, post-vaccine reaction rates are low and often benign including allergic subjects to the vaccine antigens [49–51].

Without attempting to compare the antigenicity of calcium phosphate with alum salts, these studies demonstrate the effectiveness of calcium phosphate as adjuvant for mentioned vaccines. Calcium phosphate is therefore likely to be reused in human vaccination without discrimination of individuals allergic to toxoids.

Indeed, anti-polio vaccine based on calcium phosphate allows sustainable vaccination of total polio-unimmunized persons [50]. Furthermore, associations of antigens diphtheria, tetanus, pertussis, and polio, possible with calcium phosphate, have allowed the simplification of the vaccination schedule [49]. Rare and mild local reactions make that kind of vaccines well tolerated, including for persons previously allergic to antigens, for which vaccination was until contraindicated [50,51]. The generally lower antigenicity of calcium phosphate could be actually a reflection of its lower capacity adsorption than aluminum adjuvants with certain antigens and in certain preparation conditions [52]. Despite its lower antigenicity than

alum salts, calcium phosphate has demonstrated its effectiveness making it usable in vaccination [31,53].

On the other hand, it seems obvious for some authors that the improved vaccine safety comes at the expense of immunogenicity resulting from a compromise between antigenicity and safety [54,55]. The inflammatory or danger-signal model of adjuvant action implies that increased vaccine reactogenicity is the inevitable price for improved antigenicity. Hence, adjuvant reactogenicity may be avoidable only if it is possible to separate inflammation from adjuvant action [56]. This is an encouraging observation for the nanoparticulate calcium phosphate because inflammasome activation could not be required for its antigenicity [37]. Other factors such as the age of the vaccinated organism could play a role in vaccine and adjuvant effectiveness [57]. Similarly, data obtained using animal model may not always be reported to humans, as reflected in the saying that ‘mice lie’ [56]. Human trials would be more appropriate to provide information specifically about the ability of calcium phosphate to induce an appropriate immune response.

Under well-defined conditions, highly purified toxoids adsorbed on calcium phosphate gel produce a highly immunogenic vaccine to induce great immune response [1,20]. A study onto various allergens adsorbed on calcium phosphate (dust mites, pollens, and cat hairs) in both adults and children allowed a very good response at the level of desensitization and especially a lack of secondary reactions. Indeed, allergens adsorbed on calcium phosphate were considered safe and effective [21].

To conclude, antigenicity of calcium phosphate is size dependent, and the optimal size range would be 100–400 nm. The preparation form affects antigenicity, and the gel form should be preferentially used. Finally, beyond the comparison with alum salts, calcium phosphate has demonstrated its effectiveness for use in human vaccination although some additional analyses are needed.

3. Antigenicity, immunoglobulins, and antibody

3.1. Immunoglobulins and antibody

Immunoglobulin (Ig) and antitoxin production as antigenicity depends on many factors such as antigen, way of preparation, and particle size. Thus, tetanus toxoid adsorbed on calcium phosphate or aluminum hydroxide used in Guinea pigs showed that calcium phosphate is less efficient.

Indeed, the production of IgG 1a and 1b is lower with calcium phosphate compared to aluminum hydroxide [58]. However, the same tetanus toxoids or diphtheria toxoids adsorbed on tricalcium phosphate at higher concentration induce in both animals and humans similar or greater IgG production than what is induced by aluminum adjuvants, especially after the booster [29,47,59]. The use of different forms of calcium phosphate (hydroxyapatite, moneite, and tricalcium phosphate) at different doses makes complex the comparison of the results of these studies. It is difficult to say if the concentration or the type of compound is the most impacting on the antibody response, but both parameters must have an impact on this factor. As a natural constituent

of the human organism [15], calcium phosphate provides a slow release of adsorbed antigens at the injection site. This slow release induced high antibody production with calcium phosphate gel [41]. In humans, the concentration of anti-tetanus antibodies is equal after calcium phosphate or aluminum vaccine injection. On the other hand, 3.5 months after the booster, antibody concentration is much greater in people who have been vaccinated with calcium phosphate vaccines. Furthermore, the number of not reacting subjects after two injections is lower with calcium phosphate-based vaccines [24]. It was also possible to make successful multi-antigen vaccines on calcium phosphate (diphtheria, tetanus, polio, Bacille Calmette Guérin (BCG), yellow fever, and measles) as well as simultaneous injections, including a hepatitis B vaccine [17,60].

In addition, with the adjuvant concentration, animal model, antigen used, and others, an important factor can also operate to explain the difference of antibody production between studies, the particle size. As already mentioned in Section 2.3, small particles (20–200 nm) are preferentially caught via endocytosis by dendritic cells, and larger particles (<5 µm) are phagocytosed by macrophages, leading to efficient coadministered antigen presentation and subsequent adaptive immune response [45]. This size-dependent antigenicity has been shown by various particulates. Some adjuvants have a nanometric optimal size, and others have a micrometric optimal size, so it seems that an optimal size range exists for each particulate adjuvant. Calcium phosphate particles of 100–400 nm present the greater antibody production and the most virus neutralization for influenza in mice [37].

Although animal model results are divergent depending on antigen and adjuvant concentrations, human data suggest that calcium phosphate is an effective adjuvant and potentially more effective than alum after the booster. It has a reasonable capacity to adsorb antigens, induces high levels of IgG antibodies, and does not increase IgE production [15,17,61].

3.2. Immunoglobulin E

Vaccine adjuvants generally cause the preferential synthesis of one or more antigen group [62]. Calcium phosphate gel has a major advantage because it does not induce an increase of the rate of IgE during its use in booster in both human and animals, contrary to aluminum hydroxide [58,63,64].

The extended production of IgE could cause allergy in case of regular injections [58]. Calcium phosphate induces a very low IgE production probably because it is a natural constituent of the body [15]. The use of a higher concentration of calcium phosphate may also lead to the IgE production, but only at concentrations higher than those used for human vaccination [65].

Calcium phosphate formulation also plays a role in the IgE response. Used in humans or in mice, monetite (CaHPO_4) or tricalcium phosphate may induce low IgE levels like aluminum hydroxide in function of adsorbed antigen, while this effect is not found in Guinea pigs [47,59]. Tricalcium phosphate is defined by the authors as more effective but does not

represent a reliable alternative to aluminum hydroxide in terms of their comparable IgE production in study conditions [47,58].

In any event, the presence of specific IgE antibodies in human serum must be regarded as a potential negative effect which can appear also after booster immunization, especially for hyperimmunized and allergic persons [58]. Human IgE production is conditioned by a hereditary factor; however, many authors associate the increasing frequency of allergic diseases with the intensive mass immunization programs without regard for the possible mechanisms [66,67].

Indeed, the IgE presence in the body could stimulate an IgE response to other immunogens which come into contact with the immune system of the body at the same time. This might result in the development of an IgE response to different environmental antigens and in some cases lead to clinically manifested reactions of hypersensitivity [58], while the IgE production by aluminum vaccine has been known for a long time [68–71].

4. Innocuity

Like most adjuvants, calcium phosphate may cause small under-skin nodules, which can be palpable for 30 days after injections. These nodules then tend to be resorbed leaving a final scarry lesion with clumps of calcium deposit surrounded by a thin and mostly acellular fibrous layer [72–74]. However, the under-skin nodules may be avoided with deeper intramuscular injection [50].

It should be noted that calcium phosphate is a compound classified as safe and biocompatible by the FDA [39,75], due to its natural occurrence in the organism. While having similar properties with alum salts, calcium phosphate has the advantage that it is a natural compound to the human organism and may therefore be exceptionally well tolerated. Calcium phosphate particles are biodegradable material caught via endocytosis by macrophage or dendritic cells and degraded in lysosome [37,76]. Products of biodegradation (Ca^{2+} and PO_4^{3-}) are also physiologically present in blood in relatively high concentrations [39,77,78]. Nonbiodegradable particles such as aluminum hydroxide, widely used for human vaccines, can be retained for a long time, raising the question of biopersistence and biotranslocation, thus inducing potential toxicity (for review, see Gherardi et al. [79]). Thus, biodegradable particles are supposed to be less toxic than nonbiodegradable particles [80]. The excellent biocompatibility of calcium phosphate makes it a biomaterial used in orthopedics and dental prostheses [75].

4.1. Inflammatory reaction

The route of administration of vaccines, particularly those adsorbed onto mineral adjuvants such as calcium phosphates or alum salts, plays an important part to avoid adverse local reactions [81]. In subcutaneous injections, calcium phosphate induced active inflammation involving neutrophils and macrophages [42]. It should be noted that the subcutaneous way is now replaced by the intramuscular way because

subcutaneous injections induce nodules which are not observed when the vaccine is administered intramuscularly [50,81]. Used in Guinea pigs, intramuscular injections of calcium phosphate suspension cause histopathological effects similar to those of aluminum hydroxide [42]. However, the gel form causes more moderate reactions compared to the suspension form or to aluminum adjuvants [42,58]. It seems likely that gel form is not easily caught by phagocytes. This may explain the fact that the inflammation due to the suspension form is severer than that induced by the gel form.

The calcium phosphate gel inflammatory reaction does not last more than 4 weeks, contrary to the one induced by aluminum adjuvants that can last twice as long [42]. Some local reactions following vaccination, such as severe pain, while not directly damaging to physical health, may still have a strong negative impact on the public's perception of the risk–benefits of immunization and thus should be limited [56].

The shorter inflammatory reaction observed with calcium phosphate gel may be explained by an easier dissemination of calcium phosphate that is a natural constituent of the human body [15]. Unlike aluminum adjuvants, calcium phosphate is not toxic *in vitro* for macrophages at the concentration range used in vaccination [42,82].

This is an important point to consider calcium phosphate as an adjuvant for human vaccination and for specific immunity because the role of macrophages is recognized in the induction of immune response [62]. Moreover, the inflammatory reaction remains short even at high concentration of calcium phosphate [42]. This makes the use of calcium phosphate vaccine possible, even in case of overconcentration, a condition potentially required depending on the antigen used.

4.2. Antigen–adjuvant interaction and innocuity

Histological reactions are not equal in the presence of single adjuvant, single antigen, or combinations of both. Predicting the vaccine local reaction seems complicated as antigen adsorbed on calcium phosphate influences the overall safety of the vaccine.

For instance, in rabbits, when considered individually, calcium phosphate as in tetanus toxoid injection cause a moderate reaction with respective presence of a granuloma or vascular inflammation, which can cause small bruises. However, the combination of two molecules may induce an intense granulocytic reaction from the sixth hour accompanied by cytolysis of polynuclear leukocytes in the twenty-fourth hour.

The association of three antigens on calcium phosphate caused a relatively similar reaction to tetanus toxoid adsorbed alone on calcium phosphate in rabbits, but the addition of pertussis antigen caused a granulocytes influx to the third day, which may lead to a lesion similar to acute fibrinous inflammation [73].

Moreover, the vaccine preparation quality is crucial to ensure safety. Indeed, some of the local vaccine effects may directly reflect chemical irritation due to a non-physiological pH or osmolality and salt concentrations toxic for cells [56].

4.3. Hemolytic activity

Calcium phosphate gel shows high hemolytic activity in human, mouse, sheep, and rabbit blood samples taken after vaccination. Indeed, analyses show a high rate of free hemoglobin, indicating the erythrocytes lysis *in vitro*. This activity is reduced by 50% when the adjuvant is used in suspension form [82].

The hemolytic effect is dose dependent and remains equal to the one induced by aluminum compounds at concentration less than 1.3 mg/mL of calcium, leading to the establishment of a superior limit of 1.3 mg of calcium phosphate per vaccine dose by the WHO and the European Pharmacopoeia [26,39,40,59].

If calcium phosphate concentration is higher than 1.3 mg, the hemolytic effect of calcium phosphate becomes higher than that produced by aluminum hydroxide probably because this concentration might disrupt the delicate chemical balance of the organism. However, it is possible to greatly reduce the calcium phosphate hemolytic effect by the addition of phosphate ions to the solution. This addition also decreases the adsorption capacity of the adjuvants [40,83]. However, adjustments of adjuvant and phosphate ions concentrations allow to obtain aluminum hydroxide and calcium phosphate solutions with the same adsorption capabilities and hemolytic effect [40].

It seems important to point out that these results come of *in vitro* experiments and that they are not unanimously accepted. Indeed, calcium phosphate, while exhibiting a proved hemolytic activity, is not designed, as an adjuvant, to be injected into vascular vessels but intramuscularly or subcutaneously injected, thus leading to consider that kind of data as invalid from a clinical point of view [84]. It therefore seems important to respect strictly intramuscular injection mode to avoid hemolytic reaction at the injection site [84]. This example perfectly illustrates the fact that available safety data on a vaccine are strictly specific to the vaccine and its way of administration [19].

4.4. Nano-particular form and innocuity

Recent studies present data on the safety of calcium phosphate nanoparticles. Keeping in mind that this specific form does not probably have the same characteristics as microparticles, we wanted to add recent data to the present review.

Among adjuvant developments, many researches now focus on nanoparticle compounds. Indeed, their small size provides a high surface area to volume ratios [39]. Apart from its excellent storage stability, cold preparation, and relatively inexpensive development, calcium phosphate nanoparticles have positive characteristics in all areas studied [85,86].

In mice, calcium phosphate nanoparticles with herpes or Epstein–Barr viruses induce a sustainable total IgG, IgG2a, and IgA production without producing IgE. No inflammation evidence at the injection sites and no sign of toxicity have been shown in this study [85–87]. In studies on cultured fish, bird, and human cells, this nanoparticle adjuvant was readily absorbed and dissolved in lysosomes prior to being evacuated out of the cells [86,88–90].

It was suggested to develop a single-dose vaccine with sustained-release capabilities [85]. This form is effective for vaccination through mucosal way (intranasally and intravaginally), allowing systemic vaccination [85,87].

Alum salts selectively stimulate humoral immune responses, especially type-2 helper (Th2) immune responses, which are characterized by the production of interleukin (IL)-4 and IL-5 and the induction of IgE and IgG1 [91–93]. Calcium phosphate nanoparticles exist as rod shaped and sphere shaped. Rod-shaped one can induce both cellular immune response (development of T lymphocytes) by type-1 helper (Th1) way and humoral immune response by Th2 way [37,94,95]. Microparticle-sized adjuvants may not be able to induce the same response due to a greater size [87,96–99]. Nanoparticle adjuvants also allow the optimization of a selective cell response for the antigen release [39,100].

However, in mice, a comparison of two tetanus vaccines based on calcium or aluminum phosphate nanoparticle allowed to highlight the presence of histological lesions in brain, kidney, liver, and injected muscle. These lesions included hepatocellular necrosis infiltrated by mononuclear cells, hydropic degeneration, and appearance of Küppfer cells. They are generally less acute with the calcium phosphate except for muscle damage showing signs of hemorrhage [101].

The nanoparticle compounds are currently too new to ensure a sufficient view on their safety. These compounds, unlike the microparticle form, have higher permeability through cell membranes and thus influence the physiology of most cells and translocation to other distant organs [101,102]. This high membrane permeability requires further studies to bring out the advantages and disadvantages for vaccination [101,103,104].

4.5. Old studies, *in situ* data

The interest for calcium phosphate greatly decreased after discontinuation of its use in the 1980s, and thus, research on this molecule was progressively reduced. At present, there are only a few studies on the safety of calcium phosphate microparticles used as adjuvant, and the most recent was published at the end of the 1990s [42]. To the best of our knowledge, calcium phosphate does not seem to show evidence of any activity likely to cause allergic problems or reactions, making it incompatible with its use for human or animal vaccination. On the contrary, calcium phosphate seems to be very well tolerated [17,25,42,51,58,105,106].

Furthermore, calcium phosphate was used in France until the mid-1980s, mainly for the diphtheria–pertussis–tetanus vaccine group, without any mention of adverse reactions by physicians. It has been successfully used in the pentavalent human vaccination (smallpox, yellow fever, measles, BCG, and tetanus), without any reported adverse reaction [60].

According to several authors, calcium phosphate is a natural constituent of the human organism, thus providing a greater guarantee for the absence of adverse effects, and may be considered as safer than aluminum hydroxide [107]. Other authors recognize that an ascertained safety profile makes it interesting for the evaluation of new vaccines, but keeping in mind that as with adjuvanticity, studies conducted

on animals to test the safety of vaccines and of their adjuvants do not guarantee that they are a perfect reflection of the human situation and would thus require new studies [31,56].

5. Perspectives

Within the scientific literature on the calcium phosphate as a vaccine adjuvant, some publications highlight several perspectives for its use, potentially deserving further investigations.

In the 1990s, calcium phosphate was studied for the development of an anti-HIV vaccine in rabbits [108]. Thanks to the adsorption of the HIV-1 gp160 antigen on calcium phosphate gel [25], the final vaccine allowed a good immune response in rabbits. Although not leading to the production of anti-HIV vaccine in humans, these studies highlighted the calcium phosphate capabilities as adjuvant for the development of future promising vaccines [108,109].

Calcium phosphate has been the subject of recent research for the development of new vaccines. Another study shows the possible use of calcium phosphate in the hepatitis B DNA vaccine development on murine model [83]. This vaccine induces protective humoral- (through antibodies) and cell-mediated immune responses (through CD8 T lymphocytes) [83].

Calcium phosphate nanoparticles remain a development requiring further research. Indeed, this new formulation may present several advantages such as the adsorption capacity, duration of the immune response, and induction of cellular response or mucosal immunity, but their different adverse effects are not fully characterized or understood [39,85,87,89].

Finally, in association with mesoporous silica, calcium phosphate has been studied to develop a cancer vaccine on murine model [110]. Cancer vaccines allow the activation of immune cells, which specifically recognize and destroy tumor cells without harming normal cells. The use of cancer vaccines attempts to harness the specific power of the immune system for cancer treatment. The crucial point for cancer vaccines is the inclusion of an appropriate adjuvant [111]. In this role, the combination of mesoporous silica with calcium phosphate or Zn- and Mg-tricalcium phosphates allows a higher potent systemic antitumor immunity, which inhibits the growth of tumor both *in vitro* and *in vivo* [110,111].

In addition, since the end of the 1990s, the allergens adsorbed on the Pasteur Institute calcium phosphate were taken over by the Stallergènes laboratory and are still very widely disseminated at the international level, such as in some specific desensitization treatments by Stallergènes-Greer.

6. Expert commentary

Before considering a compound for use as an adjuvant in human vaccines, it is necessary to consider several factors, mainly to determine experimentally if the preparation is suitable and safe. These factors include safety by tests of acute and chronic toxicity, local and general tolerance teratogenicity, biodegradability, carcinogenicity or co-carcinogenicity, the degree of immunopotentiality, unacceptable or pharmacologic adverse effects, and the definition of action [62]. Calcium phosphate has successfully passed these various tests including high efficiency in terms of concentration of

Table 4. Summary of advantages and disadvantages of the use of calcium phosphate (CaP) as an adjuvant in human vaccines.

Advantages	Disadvantages
• CaP was used as an adjuvant up to 1985; data are available to use it again in a fast but not expensive way compared to the development of new adjuvants • CaP is present in the general monographic on human vaccines of the European Pharmacopoeia 8.0 • CaP is classified as safe and biocompatible by the US FDA • CaP is a natural compound of the human body, thus suggesting its good tolerance for individuals • Adsorption capability is equivalent to aluminum adjuvants, depending on preparation mode, considered antigens, and particle size • Booster antigenicity is potentially better than with aluminum adjuvants • CaP is used in allergic desensitization • CaP does not induce immunoglobulin E production that may cause an allergic reaction • Inflammatory reaction is moderate and shorter with CaP than with aluminum adjuvants • CaP does not lead to macrophagic toxicity • Good tolerance of CaP is highlighted by the lack of negative return during its commercial use	• Several forms of preparations exist: suspension and gel form (the suspension form does not appear suited for vaccinal use), micro- and nano-metric size, some formulation (see Table 1), and sphere shape or rod shape • Sensitivity of the quality of the gel form depends on the conditions of preparation • Global primo-injection antigenicity is weaker than with aluminum adjuvants • Due to severe hemolytic effect with intravascular injection, intramuscular injection is imperative

circulating antibody, which allowed its use in several human vaccines for 20 years [1,4].

In the development of novel adjuvants for vaccination, several tracks are promising but represent expensive and long-lasting research. The immediate use of calcium phosphate is therefore desirable because the information collected for human vaccination has demonstrated that its use is possible. The lack of data on this molecule does not allow a strict conclusion about its safety. However, the available human data suggest an adequate ability of calcium phosphate to produce an effective immune response with diphtheria, tetanus, polio, and pertussis antigens in certain defined conditions [25]. Calcium phosphate is a compound naturally present in the organism, characterized as safe by the FDA. To the best of our knowledge, no study has revealed adverse effects when used under clinical conditions; thus, no reason seems to justify the sidelining of this adjuvant in human vaccination for cited antigens. This is confirmed by its use in specific desensitization for more than 30 years.

To conclude, beyond the knowledge of differences between calcium phosphate and aluminum salts, and comparisons to determine if calcium phosphate is more or less efficient than aluminum salts, we showed in this review that calcium phosphate and specially the hydroxyapatite form represents a good candidate to replace or to complete alum salts as a vaccine adjuvant (Table 4). The calcium phosphate use as nonspecific adjuvant for other antigens should be subjected to further research, focusing particularly on the response of the compound gel form that has already been used in humans. This compound is present in the International Pharmacopoeia and is part of the additives approved by the WHO. It is also mentioned as a possible adjuvant in the European Pharmacopoeia. In addition, the president of French Technical Committee of Vaccinations admitted that calcium phosphate is as effective as aluminum. Finally, the patent of calcium phosphate is now in the public domain, making it accessible for further utilization in different future vaccines.

7. Five-year view

Research on calcium phosphate should be resumed. In the next 5 years, studies and clinical trials necessary for the

calcium phosphate reuse in human vaccination could be completed. This would offer a greater range of vaccines. This wider range would enable patients to choose an adjuvant. Patients who do not want to be vaccinated with aluminum adjuvants would thus have access to immunization with calcium phosphate, thus ensuring a great vaccinal coverage.

Key issues

- Calcium phosphate was used as a vaccine adjuvant.
- It was replaced by aluminum salts at the end of 80's.
- Aluminum salts raise safety questions.
- Calcium phosphate can be as effective as aluminum salts.
- It is a natural compound of the human body.
- Human data highlight the good tolerance of calcium phosphate.
- The widespread use of calcium phosphate requires further studies.

Acknowledgments

We are grateful to E & R Jandot for their careful revision and critical comments on this paper.

Funding

This work has been carried out with E3M association with the financial support of Mutuelle Familiale.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Documentary research methodology

The aim of this study has been to make an international state of the art about the use of calcium phosphate as an adjuvant in vaccine. Exhaustive documentary research has been conducted in order to establish a

quantitative and qualitative usable data assessment. All published data concerning advantages and disadvantages of the use of calcium phosphate have been sought. Points of View or recommendations on the inclusion of the calcium phosphate in vaccines or more broadly on the adjuvant safety have been identified. Documentary sources database, grey literature, relevant internet sites as well as (French) regulatory information have been sorted, assessed and interviewed in a systematic way. The recommendations, meta-analysis and systematic reviews have been sought in Medline from key words: ('Adjuvants, Immunologic'[Mesh] OR Adjuvant*) AND (vaccine* OR immunol*)[title] then scientific publications on the use of calcium phosphate as adjuvant from key words: 'Calcium phosphate' [Supplementary Concept] OR 'Calcium Compounds'[Mesh] OR 'Calcium Phosphates'[Mesh] OR calcium phosphate OR CaP Field: Title AND ('Adjuvants, Immunologic'[Mesh] OR adjuvant* Field: Title). 160 references, with various quality and relevance, have been obtained on the basis of this research and a hundred of them have proved workable.

References

Papers of special note have been highlighted as either of interest (*) or of considerable interest (**) to readers.

- Relyveld EH, Levaditi J-C, Ravisce P. Adjuvants minéraux. *Médecine Mal Infect.* 1978;8:494–499.
- De Souza Apostólico J, Lunardelli VAS, Coirada FC, et al. Adjuvants: classification, modus operandi, and licensing. *J Immunol Res.* 2016;2016:1459394.
- Ramon G. Sur l'augmentation anormale de l'antitoxine chez les chevaux producteurs de sérum antidiphtériques. *Bull Soc Centr Med Vet.* 1925;101:227–234.
- Bégué P, Girard M, Bazin H, et al. Les adjuvants vaccinaux: quelle actualité en 2012? *Bull Acad Natle Med.* 2012;196:1177–1181.
- Glenny AT, Pope CG, Waddington H, et al. Immunological notes. XVII–XXIV. *J Pathol Bacteriol.* 1926;29:31–40.
- Tomljenovic L, Shaw CA. Aluminum vaccine adjuvants: are they safe? *Curr Med Chem.* 2011;18:2630–2637.
- Gherardi RK, Coquet M, Cherin P, et al. Macrophagic myofasciitis: an emerging entity. *The Lancet.* 1998;352:347–352.
- World Health Organization. Macrophagic myofasciitis and aluminium-containing vaccines. *Wkly Epidemiol Rec.* 1999;74:338–340.
- Exley C, Swarbrick L, Gherardi RK, et al. A role for the body burden of aluminium in vaccine-associated macrophagic myofasciitis and chronic fatigue syndrome. *Med Hypotheses.* 2009;72:135–139.
- Exley C. Human exposure to aluminium. *Environ Sci Process Impacts.* 2013;15:1807–1816.
- Exley C. Why industry propaganda and political interference cannot disguise the inevitable role played by human exposure to aluminium in neurodegenerative diseases, including Alzheimer's disease. *Front Neurol.* 2014;5:212.
- Aprile MA, Wardlaw AC. Aluminium compounds as adjuvants for vaccines and toxoids in man: a review. *Can J Public Health.* 1966;57:343–360.
- Exley C. What is the risk of aluminium as a neurotoxin? *Expert Rev Neurother.* 2014;14:589–591.
- Relyveld EH. Calcium phosphate gel for adsorbing vaccines. 4016252. 1977.
- Relyveld EH, Henocq E, Raynaud M. Etude de la vaccination antidiphtérique de sujets allergiques, avec une anatoxine pure adsorbée sur phosphate de calcium. *Bull World Health Organ.* 1964;30:321.
- Coursaget P, Yvonnet B, Relyveld EH, et al. Simultaneous administration of diphtheria-tetanus-pertussis-polio and hepatitis B vaccines in a simplified immunization program: immune response to diphtheria toxoid, tetanus toxoid, pertussis, and hepatitis B surface antigen. *Infect Immun.* 1986;51:784–787.
- Gupta RK, Rost BE, Relyveld E, et al. Adjuvant properties of aluminium and calcium compounds. *Pharm Biotechnol.* 1995;6:229–248.
- .. This book chapter explains two different ways of preparation of calcium phosphate and discusses the different properties of obtained adjuvant.**
- Moulin A-M. L'Aventure de la vaccination. Paris: Fayard; 1996.
- Sesardic D, Rijpkema S, Patel BP. New adjuvants: EU regulatory developments. *Expert Rev Vaccines.* 2007;6:849–861.
- Relyveld EH, Raynaud M. Etudes sur le phosphate de calcium comme adjuvant de l'immunité. *Int. Symp. Adjuv. Immun., vol. 6, Karger Heidelberg.* New York; 1967. p. 77–78.
- Lecadet A, Ickovic MR, Thibaudon M. Specific desensitization with allergen extracts absorbed on calcium phosphate (Pasteur Institute). *Clinical and biological study apropos of 107 cases.* *Allerg Immunol (Leipz).* 1988;20:153–155.
- Relyveld EH. Etudes sur la toxine et l'antitoxine diphtériques et la réaction toxine-antitoxine [Thèse Paris]. Paris: Editions scientifiques Hermann; 1958.
- Raynaud M, Relyveld EH, Turpin A, et al. Preparation of highly purified diphtheric, tetanic & staphylococcal anatoxins absorbed on calcium phosphate (brushite). *Ann Inst Pasteur.* 1959;96:60–71.
- .. This article is the first presentation of preparation mode of calcium phosphate for adjuvant use.**
- Relyveld E, Bengounia A, Huet M, et al. Antibody response of pregnant women to two different adsorbed tetanus toxoids. *Vaccine.* 1991;9:369–372.
- Relyveld EH. Preparation and use of calcium phosphate adsorbed vaccines. *Dev Biol Stand.* 1986;65:131–136.
- Jiang D, Premachandra GS, Johnston C, et al. Structure and adsorption properties of commercial calcium phosphate adjuvant. *Vaccine.* 2004;23:693–698.
- Dixon JB, Weed SB. Minerals in soil environments. Madison, WI: Soil Science Society of America (SSSA); 1989.
- Bleam WF, Pfeffer PE, Goldberg S, et al. A phosphorus-31 solid-state nuclear magnetic resonance study of phosphate adsorption at the boehmite/aqueous solution interface. *Langmuir.* 1991;7:1702–1712.
- Seeber SJ, White JL, Hem SL. Predicting the adsorption of proteins by aluminium-containing adjuvants. *Vaccine.* 1991;9:201–203.
- Al-Shakhshir R, Regnier F, White JL, et al. Effect of protein adsorption on the surface charge characteristics of aluminium-containing adjuvants. *Vaccine.* 1994;12:472–474.
- Singh M, Ugozzoli M, Kazzaz J, et al. A preliminary evaluation of alternative adjuvants to alum using a range of established and new generation vaccine antigens. *Vaccine.* 2006;24:1680–1686.
- Lindblad EB. Aluminium compounds for use in vaccines. *Immunol Cell Biol.* 2004;82:497–505.
- Aggerbeck H, Heron I. Adjuvanticity of aluminium hydroxide and calcium phosphate in diphtheria-tetanus vaccines—I. *Vaccine.* 1995;13:1360–1365.
- Sivananda N, Sundaran B. Studies on adsorption of diphtheria toxoid on aluminium phosphate gel. *Indian J Sci Technol.* 2010;3:248–249.
- Iyer S, HogenEsch H, Hem SL. Effect of the degree of phosphate substitution in aluminum hydroxide adjuvant on the adsorption of phosphorylated proteins. *Pharm Dev Technol.* 2003;8:81–86.
- Iyer S, Robinett RSR, HogenEsch H, et al. Mechanism of adsorption of hepatitis B surface antigen by aluminum hydroxide adjuvant. *Vaccine.* 2004;22:1475–1479.
- Hayashi M, Aoshi T, Kogai Y, et al. Optimization of physiological properties of hydroxyapatite as a vaccine adjuvant. *Vaccine.* 2016;34:306–312.
- Ginebra MP, Driessens FCM, Planell JA. Effect of the particle size on the micro and nanostructural features of a calcium phosphate cement: a kinetic analysis. *Biomaterials.* 2004;25:3453–3462.
- Rivera Gil P, Hühn D, Del Mercato LL, et al. Nanopharmacy: inorganic nanoscale devices as vectors and active compounds. *Pharmacol Res.* 2010;62:115–125.
- Kato H, Shibano M, Saito T, et al. Relationship between hemolytic activity and adsorption capacity of aluminum hydroxide and calcium phosphate as immunological adjuvants for biologicals. *Microbiol Immunol.* 1994;38:543–548.
- Olmedo H, Herrera M, Rojas L, et al. Comparison of the adjuvant activity of aluminum hydroxide and calcium phosphate on the

- antibody response towards *Bothrops asper* snake venom. *J Immunotoxicol.* 2014;11:44–49.
- **This article highlights the good adsorption capacity of calcium phosphate for several fraction of snake venom. In this study, venom adsorbed on calcium phosphate induced a high anti-body response.**
42. Goto N, Kato H, Maeyama J, et al. Local tissue irritating effects and adjuvant activities of calcium phosphate and aluminium hydroxide with different physical properties. *Vaccine.* 1997;15:1364–1371.
 - **This article presents the irritating effect of aluminum hydroxide and calcium phosphate adjuvant to local tissue. The more interesting result is the duration of inflammatory reaction twice as short with the calcium phosphate as aluminum hydroxide.**
 43. Kuroda E, Coban C, Ishii KJ. Particulate adjuvant and innate immunity: past achievements, present findings, and future prospects. *Int Rev Immunol.* 2013;32:209–220.
 44. Zhao L, Seth A, Wibowo N, et al. Nanoparticle vaccines. *Vaccine.* 2014;32:327–337.
 45. Shah RR, O'Hagan DT, Amiji MM, et al. The impact of size on particulate vaccine adjuvants. *Nanomed.* 2014;9:2671–2681.
 46. Ibrahim-Saeed M, Omar AR, Hussein MZ, et al. Systemic antibody response to nano-size calcium phosphate biocompatible adjuvant adsorbed HEV-71 killed vaccine. *Clin Exp Vaccine Res.* 2015;4:88–98.
 47. Aggerbeck H, Fenger C, Heron I. Booster vaccination against diphtheria and tetanus in man. Comparison of calcium phosphate and aluminium hydroxide as adjuvants-II. *Vaccine.* 1995;13:1366–1374.
 48. Donat N, Thibaudon M, Peltre G, et al. Desensitization for 4 successive years using calcium phosphate-adsorbed pollen extracts: study of sera with RAST and PRIST immunoimprints. *Allerg Immunol (Leipz).* 1986;18:24–31.
 49. Martin R, Relyveld EH, Raynaud M, et al. Immunizing strength and tolerance by the human organism of vaccines adsorbed on calcium phosphate. *Presse Médicale.* 1969;77:341–344.
 50. Relyveld EH, Martin R, Raynaud M, et al. Calcium phosphate as adjuvant in vaccinations in man. *Ann Inst Pasteur.* 1969;116:300–326.
 51. Relyveld EH, Martin R, Raynaud M, et al. Vaccination with calcium phosphate adsorbed antigens. *Prog Immunobiol Stand.* 1970;4:540–547.
 52. Edelman R. Vaccine adjuvants. *Rev Infect Dis.* 1980;2:370–383.
 53. Wack A, Baudner BC, Hilbert AK, et al. Combination adjuvants for the induction of potent, long-lasting antibody and T-cell responses to influenza vaccine in mice. *Vaccine.* 2008;26:552–561.
 54. Gupta RK, Relyveld EH, Lindblad EB, et al. Adjuvants a balance between toxicity and adjuvanticity. *Vaccine.* 1993;11:293–306.
 55. Poolman JT. Shortcomings of pertussis vaccines: why we need a third generation vaccine. *Expert Rev Vaccines.* 2014;13:1159–1162.
 56. Petrovsky N. Comparative safety of vaccine adjuvants: a summary of current evidence and future needs. *Drug Saf.* 2015;38:1059–1074.
 57. Mastelic B, Ahmed S, Egan WM, et al. Mode of action of adjuvants: implications for vaccine safety and design. *Biologicals.* 2010;38:594–601.
 58. Vassilev TL. Aluminium phosphate but not calcium phosphate stimulates the specific IgE response in guinea pigs to tetanus toxoid. *Allergy.* 1978;33:155–159.
 - **This comparison of aluminum phosphate and calcium phosphate antigenicities demonstrates that booster immunization with aluminum but not calcium phosphate-adsorbed toxoid leads to a prolonged synthesis of specific IgE. This article is an extension of older study mentioned.**
 59. Gupta RK, Siber GR. Comparison of adjuvant activities of aluminium phosphate, calcium phosphate and stearyl tyrosine for tetanus toxoid. *Biol J Int Assoc Biol Stand.* 1994;22:53–63.
 60. Gateff C, Relyveld EH, Le Gonidec G, et al. Study of a new pentavalent vaccine combination. *Ann Microbiol (Paris).* 1973;124:387–409.
 61. Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. *Immunol Cell Biol.* 2004;82:488–496.
 62. Organisation Mondiale de la Santé. Adjuvants de l'immunité. *Sér Rapp Tech.* 1976;595:43.
 63. Relyveld EH, Laverne M, De Rudder J. Titers of immunoglobulins (IgG, IgA, IgM, and IgE) after antigenic stimulation in European and African subjects. *Dev Biol Stand.* 1974;27:79–90.
 64. Ickovic MR, Relyveld EH, Hénocq E, et al. Calcium-phosphate-adjuvanted allergens: total and specific IgE levels before and after immunotherapy with house dust and *Dermatophagoides pteronyssinus* extracts. *Ann Immunol.* 1983;134D:385–398.
 65. Parish WE. Homologous serum passive cutaneous anaphylaxis in Guinea pigs mediated by two $\gamma 1$ or $\gamma 1$ -type heat-stable globulins and a non- $\gamma 1$ heat-labile reagent. *J Immunol.* 1970;105:1296–1298.
 66. Relyveld EH. Immunoglobulins and reaginic activity. *Rev Fr Allergol.* 1969;9:219–232.
 67. Hubscher T. Immune and biochemical mechanisms in the allergic disease of the upper respiratory tract; role of antibodies, target cells, mediators and eosinophils. *Ann Allergy.* 1977;38:83–90.
 68. Kishimoto T, Ishizaka K. Regulation of antibody response in vitro. VII. Enhancing soluble factors for IgG and IgE antibody response. *J Immunol Baltim Md 1950.* 1973;111:1194–1205.
 69. Nagel J, Svec D, Waters T, et al. IgE synthesis in man. I. Development of specific IgE antibodies after immunization with tetanus-diphtheria (Td) toxoids. *J Immunol Baltim Md 1950.* 1977;118:334–341.
 70. Mitani S, Yamamoto A, Ikegami H, et al. Immunoglobulin E-suppressing and immunoglobulin G-enhancing tetanus toxoid prepared by conjugation with pullulan. *Infect Immun.* 1982;36:971–976.
 71. Cogné M, Ballet JJ, Schmitt C, et al. Total and IgE antibody levels following booster immunization with aluminum adsorbed and non-adsorbed tetanus toxoid in humans. *Ann Allergy.* 1985;54:148–151.
 72. Balouet G, Levaditi JC, Relyveld E. Le granulome immunogène: histopathologie expérimentale des lésions liées à des injections vaccinales associées à divers adjuvants de l'immunité. *Bull Inst Pasteur.* 1975;73:383–409.
 73. Balouet G, Baret M, Relyveld E, et al. Role of antigens and adjuvant substances in the histological response in experimental granulomas (immunogenic granuloma). *Ann Anat Pathol (Paris).* 1977;22:159–170.
 74. Levaditi JC, Relyveld E. Local tolerance of vaccines adsorbed on immuno-stimulating substances. *Sem Hôp Ther.* 1975;51:117–118.
 75. Dorozhkin SV, Epple M. Biological and medical significance of calcium phosphates. *Angew Chem Int Ed.* 2002;41:3130–3146.
 76. Jarcho M. Calcium phosphate ceramics as hard tissue prosthetics. *Clin Orthop.* 1981;157:259–278.
 77. Alberts B, Johnson A, Lewis J, et al. Molecular biology of the cell. 4th ed. New York: Garland Science; 2002.
 78. Wang S, McDonnell EH, Sedor FA, et al. pH effects on measurements of ionized calcium and ionized magnesium in blood. *Arch Pathol Lab Med.* 2002;126:947–950.
 79. Gherardi RK, Eidi H, Crépeaux G, et al. Biopersistence and brain translocation of aluminum adjuvants of vaccines. *Front Neurol.* 2015;6:4.
 80. Ahmed KK, Geary SM, Salem AK. Applying biodegradable particles to enhance cancer vaccine efficacy. *Immunol Res.* 2014;59:220–228.
 81. Relyveld EH, Bizzini B, Gupta RK. Rational approaches to reduce adverse reactions in man to vaccines containing tetanus and diphtheria toxoids. *Vaccine.* 1998;16:1016–1023.
 82. Goto N, Kato H, Maeyama J, et al. Studies on the toxicities of aluminium hydroxide and calcium phosphate as immunological adjuvants for vaccines. *Vaccine.* 1993;11:914–918.
 - **This study highlights the interest of calcium phosphate because of the absence of toxic effects on macrophages.**
 83. Wang S, Liu X, Fisher K, et al. Enhanced type I immune response to a hepatitis B DNA vaccine by formulation with calcium- or aluminium phosphate. *Vaccine.* 2000;18:1227–1235.
 84. Léry L. Haemolytic activity of calcium phosphate adjuvant. *Vaccine.* 1994;12:475.
 85. He Q, Mitchell AR, Johnson SL, et al. Calcium phosphate nanoparticle adjuvant. *Clin Diagn Lab Immunol.* 2000;7:899–903.

86. Koppad S, Raj GD, Gopinath VP, et al. Calcium phosphate coupled Newcastle disease vaccine elicits humoral and cell mediated immune responses in chickens. *Res Vet Sci*. 2011;91:384–390.
87. He Q, Mitchell A, Morcol T, et al. Calcium phosphate nanoparticles induce mucosal immunity and protection against herpes simplex virus type 2. *Clin Diagn Lab Immunol*. 2002;9:1021–1024.
88. Neumann S, Kovtun A, Dietzel ID, et al. The use of size-defined DNA-functionalized calcium phosphate nanoparticles to minimise intracellular calcium disturbance during transfection. *Biomaterials*. 2009;30:6794–6802.
89. Behera T, Swain P. Antigen adsorbed calcium phosphate nanoparticles stimulate both innate and adaptive immune response in fish, *Labeo rohita* H. *Cell Immunol*. 2011;271:350–359.
90. Viswanathan K, Gopinath VP, Raj GD. Formulation of Newcastle disease virus coupled calcium phosphate nanoparticles: an effective strategy for ocular delivery to chicken. *Colloids Surf B Biointerfaces*. 2014;116:9–16.
91. Gupta RK. Aluminum compounds as vaccine adjuvants. *Adv Drug Deliv Rev*. 1998;32:155–172.
92. Aimaniananda V, Haensler J, Lacroix-Desmazes S, et al. Novel cellular and molecular mechanisms of induction of immune responses by aluminum adjuvants. *Trends Pharmacol Sci*. 2009;30:287–295.
93. Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. *Nat Rev Immunol*. 2009;9:287–293.
94. Wang X, Li X, Ito A, et al. Rod-shaped and substituted hydroxyapatite nanoparticles stimulating type 1 and 2 cytokine secretion. *Colloids Surf B Biointerfaces*. 2016;139:10–16.
95. Wang X, Li X, Ito A, et al. Rod-shaped and fluorine-substituted hydroxyapatite free of molecular immunopotentiators stimulates anti-cancer immunity in vivo. *Chem Commun Camb Engl*. 2016;52:7078–7081.
96. Sokolova V, Knuschke T, Buer J, et al. Quantitative determination of the composition of multi-shell calcium phosphate-oligonucleotide nanoparticles and their application for the activation of dendritic cells. *Acta Biomater*. 2011;7:4029–4036.
97. Knuschke T, Eppler M, Westendorf AM. The type of adjuvant strongly influences the T-cell response during nanoparticle-based immunization. *Hum Vaccines Immunother*. 2014;10:164–169.
98. Zhou W, Moguche AO, Chiu D, et al. Just-in-time vaccines: biomimetic calcium phosphate core-immunogen shell nanoparticles induce long-lasting CD8(+) T cell responses in mice. *Nanomed Nanotechnol Biol Med*. 2014;10:571–578.
99. Jones S, Asokanathan C, Kmiec D, et al. Protein coated microcrystals formulated with model antigens and modified with calcium phosphate exhibit enhanced phagocytosis and immunogenicity. *Vaccine*. 2014;32:4234–4242.
100. Jilek S, Merkle HP, Walter E. DNA-loaded biodegradable microparticles as vaccine delivery systems and their interaction with dendritic cells. *Adv Drug Deliv Rev*. 2005;57:377–390.
101. Issa AM, Salim MS, Zidan H, et al. Evaluation of the effects of aluminum phosphate and calcium phosphate nanoparticles as adjuvants in vaccinated mice. *Int J Chem Eng Appl*. 2014;5:367–373.
102. Muddana HS, Morgan TT, Adair JH, et al. Photophysics of Cy3-encapsulated calcium phosphate nanoparticles. *Nano Lett*. 2009;9:1559–1566.
103. Oberdörster E. Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass. *Environ Health Perspect*. 2004;112:1058–1062.
104. Tamuly S, Saxena MK. Preparation of calcium phosphate nanoparticles and evaluation of their effects on muscle cells of rat. *Curr Sci*. 2012;102:610–612.
105. Relyveld EH, Raynaud M, Martin R, et al. Tolerance in humans of pertussis vaccines adsorbed onto calcium phosphate. *Symp Ser Immunobiol Stand*. 1970;13:171–179.
106. Sureau P, Fabre PS, N'Garro SB, et al. Vaccination simultanée de nourrissons en milieu tropical contre le tétanos et la poliomyélite. *Bull World Health Organ*. 1977;55:739.
107. McLachlan DR, Lukiw WJ, Kruck TP. New evidence for an active role of aluminum in Alzheimer's disease. *Can J Neurol Sci J Can Sci Neurol*. 1989;16:490–497.
108. Relyveld E, Chermann JC. Humoral response in rabbits immunized with calcium phosphate adjuvanted HIV-1 gp160 antigen. *Biomed Pharmacother Bioméd Pharmacothér*. 1994;48:79–83.
109. Relyveld E, Bizzini B. Production in rabbits of high levels of anti-HIV-1 gp160 antibodies. *Cell Mol Biol Noisy-Gd Fr*. 1995;41:389–393.
110. Li X, Wang X, Sogo Y, et al. Mesoporous silica-calcium phosphate-tuberculin purified protein derivative composites as an effective adjuvant for cancer immunotherapy. *Adv Healthc Mater*. 2013;2:863–871.
111. Wang X, Li X, Onuma K, et al. Zn- and Mg- containing tricalcium phosphates-based adjuvants for cancer immunotherapy. *Sci Rep*. 2013;3:2203.