Inorganic compounds as vaccine adjuvants

REVIEW

Armando A Paneque-Quevedo

Dirección de Producción, Centro de Química Biomolecular, CQB
Calle 200 y Ave. 21, Atabey, Playa, La Habana, Cuba
armando.paneque@cqb.cu, armandopaneque@infomed.sld.cu,
panecu@yahoo.com

ABSTRACT

The adjuvant capacity of minerals was first discovered in 1926, when a suspension of diphtheria toxoid precipitated with potassium aluminum sulfate was found to be significantly more immunogenic than the same suspension in the absence of this compound. Although a large number of inorganic salts have since been evaluated for this purpose, only alum, phosphate and aluminum hydroxide, and aluminum sulfate hydroxyphosphate have been approved in humans, and calcium phosphate is included in some vaccines manufactured in Europe. In the past, lack of awareness of the fact that the adjuvant properties of inorganic salts are highly dependent on nuances of their production processes that directly affect the depot and presenting effects attributed to adjuvants has led to the rejection of many compounds with potentially better adjuvant properties than traditional aluminum salts. However, the application of recent advances in nanotechnology and the combination of different adjuvants have led to the emergence and evaluation of a large number of new alternatives. The present review describes the most frequently cited inorganic adjuvants, examining their potential for the development of more potent vaccines than the current crop of products using aluminum-based compounds.

Keywords: inorganic adjuvants, aluminum salts, alum, vaccine

Biotecnología Aplicada 2013;30:250-256

RESUMEN

Compuestos inorgánicos como adyuvantes de vacunas. La propiedad adyuvante de los minerales fue descubierta en 1926, al observarse que una suspensión de toxoide diftérico precipitado con sulfato doble de aluminio y potasio proveía de una inmunogenicidad notablemente superior al toxoide sin adyuvante. Desde entonces se han evaluado numerosas sales inorgánicas como adyuvantes de vacunas y las únicas aprobadas para su uso en seres humanos son el sulfato doble de aluminio y potasio, el fosfato e hidróxido de aluminio y el sulfato de hidroxifosfato de aluminio. El fosfato de calcio se ha utilizado en algunas vacunas europeas. Las propiedades adyuvantes de las sales inorgánicas son muy dependientes del proceso de obtención e inciden sobre los efectos depósito y presentador atribuidos a estas. La no observancia de ello ha conducido al rechazo de muchas sales con mejores propiedades adyuvantes que las tradicionales sales de aluminio. La aplicación de los últimos avances de la nanotecnología y la alternativa de combinar adyuvantes han motivado la síntesis y evaluación de nuevos adyuvantes. En esta revisión se describen los adyuvantes inorgánicos citados con mayor frecuencia y sus potencialidades para el desarrollo de vacunas más eficaces que las que poseen sales de aluminio como adyuvantes.

Palabras clave: adyuvantes inorgánicos, sales de aluminio, alúmina, vacuna

*I*ntroduction

Minerals are not only the basic constituents of the earth's crust, but are important components of fertilizers, chemicals and paints, receiving intensive use in industries such as the manufacture of paper and cosmetics. They also have numerous applications in medicine, employed directly as active pharmaceutical ingredients, additives in antacid formulations and nutritional supplements; or excipients, carriers and encapsulating agents in novel formulations. Minerals are also used as adjuvants in the formulation of vaccines [1-3].

Vaccination still represents one of the most effective and safe medical interventions. From the moment of its initial discovery, it became the golden standard for disease prevention, and is regarded as the most important public health achievement in the history of humankind [4]. Vaccines are known to induce a longer and more potent immune response when an adjuvant is included in their chemical makeup [5-8].

Adjuvants are substances that, when combined with an antigen, potentiate the immune response against the latter. Not surprisingly, therefore, the inclusion of adjuvants is one of the best strategies for increasing vaccine efficacy. The word 'adjuvant' comes from the Latin *adjuvare*, that is, 'to help, to assist' [7, 8]. The adjuvant character of minerals was first discovered by Gleny *et al.* in 1926, who noticed that a suspension of diphtheria toxoid precipitated with aluminum and potassium sulphate (alum) was notably more immunogenic than the same suspension in the absence of this mineral [9].

In general, modern reviews of the state of the art in adjuvant technology tend to focus on either immunostimulating molecules (saponins-QS21, CpG oligonucleotides, lipopolysaccharides, monophosphoryl lipid A, cytokines), lipid-based structures (liposomes, virosomes, *cochleates*) or particulates (virus-like poloxamer particles), touching on the subject of traditional aluminum salts-based adjuvants only succinctly [4, 5].

In addition, the number of publications in this field focusing on other inorganic compounds has dwindled, giving the false impression that the potential applications of solid inorganic adjuvants have already been thoroughly explored, and that the time and resources of

1. Chang L. Industrial mineralogy: materials, processes, and uses. New Jersey: Prentice-Hall; 2001.

2. Carretero MI, Pozo M. Clay and non-clay minerals in the pharmaceutical and cosmetic industries. Part II. Active ingredients. Appl Clay Sci. 2010;47:171-81.

3. Reinke CM, Breitkreutz J, Leuenberger H. Aluminium in over-the-counter drugs risks outweigh benefits? Drug Safety. 2003; 26(14):1011-25.

4. Harandi AM, Gwyn D, Olesen OF. Vaccine adjuvants: scientific challenges and strategic initiatives. Expert Rev Vaccines. 2009;8(3):293-8.

5. De Gregorio E, Tritto E, Rappuoli R. Alum adjuvanticity: Unraveling a century old mystery. Eur J Immunol. 2008;38: 2068-71.

6. Singh M, O'Hagan DT. Recent advances in vaccine adjuvants. Pharm Res. 2002;19(6):715-28.

7. Clements CJ, Griffiths E. The global impact of vaccines containing aluminium adjuvants. Vaccine. 2002;20:24-33.

the scientific community would be better spent based on mechanistically different adjuvants, such as those acting as danger signals for the immune system. However, experience has shown that a one-size-fits-all adjuvant producing an optimal response to every antigen simply does not exist, otherwise very potent and promising alternatives have eventually been discarded due to issues with their stability, toxicity or production costs [10-12].

A detailed analysis of the starting materials and procedures used to obtain the constituent minerals of inorganic adjuvants suggests that it is possible, simply by accurately controlling manufacturing conditions and carefully modifying their physico-chemical properties, to obtain mineral adjuvants far superior to traditional aluminum salts-based variants, either alone or in combination with other compounds.

In this review, a comprehensive compendium is presented on the available information concerning solid inorganic compounds used as adjuvants in vaccines currently used in humans, in veterinary medicine or in biomedical research, where they are widely applied for the preparation of specific polyclonal antisera. Closer attention was paid to the structural characteristics of the most important inorganic adjuvants, as the amount of available information in the literature about the latter tends to be much larger. Other compounds of this type, which have failed to reach the stage of clinical application, are not discussed in detail, summarizing instead the results from existing studies.

Mechanism of action

Many vaccine adjuvants form depots at the injection site from which the antigen is released slowly, thereby sustaining antigen exposure to the immune system for a longer time and, in consequence, eliciting a stronger response.

For a long time, the prevailing view was that this was the sole or principal mechanism of action of immunological adjuvants [13-16]. Today, however, it is known that vaccine adjuvants act through many different and not mutually exclusive mechanisms including, in addition to physical persistence, the stimulation of inflammatory processes and cytokine release, more efficient antigen delivery to antigen-presenting cells (thanks to their particulate structure and a size smaller than 10 μm), the stimulation of immunocompetent cells through complement activation, the induction of eosinophilia at the injection site, and macrophage activation [8].

In 2008 several research teams working in the field of vaccine adjuvants proposed that the activation of the inflammasome, a protein complex performing different roles in innate immune responses [5], was another mechanism of action for adjuvants. Inflammasome activation is thought to be triggered by the particulate nature of antigen-adjuvant complexes, rather than their exact chemical composition [17, 18].

Limitations

The limitations of inorganic adjuvants include local adverse reactions such as inflammation, granulomas, abscesses and the induction of immune responses biased towards the production of immunoglobulin E (IgE), which is associated with allergic processes. They are also not the most potent inducers of antibody responses, and are not effective for the induction of the T-helper cell type 1 (Th1)-biased immune responses mediated by cytotoxic T-cell lymphocytes, which are deemed essential to provide protection against intracellular parasites [19-26].

In general, the crystalline structure of inorganic adjuvants changes when temperature decreases. It has been postulated that during solvent freezing and crystallization, colloidal adjuvant particles congeal into regions denominated 'freezing concentrates' where they are brought into close contact and repulsive interparticle forces are overcome, originating a system of coagulated or flocculated particles from which reversion to the original suspension is impossible [27]. This irreversible deterioration of the structure of adjuvant gels negatively impacts the physical stability of vaccines and, consequently, their potency [28], accounting for the fact that it is impossible to produce lyophilized vaccines when employing inorganic adjuvants. In addition, this phenomenon explains why these vaccines cannot be stored at temperatures close to 0 °C.

Adsorption

The larger and better immune responses made possible by the use of inorganic adjuvants depend on the structure, properties and mechanisms of adsorption of the latter. Among the main mechanisms postulated to explain the adsorption of antigens to inorganic adjuvants are electrostatic interactions, ligand exchange and hydrophobic interactions. In some systems, van der Waals forces as well as hydrogen bonding are also thought to play an important role in this process [29-31].

Mean particle size, morphology and surface charge have the largest impact on the adsorption of antigens to inorganic adjuvants. Adjuvants with an isoelectric point above physiological pH become positively charged at that pH, and will readily adsorb negatively charged antigens. Conversely, those with isoelectric points below physiological pH will adsorb positively charged antigens at that pH, so van der Waals forces and hydrophobic interactions will predominate whenever the isoelectric points of the antigen and adjuvant are similar. Quantitatively, adsorption will depend on the chemical makeup and concentration of the antigen, the presence of salts or ions such as those provided by commonly used buffers, and the pH of the resulting solution [32, 33].

Moreover, recent results have suggested that the process of adsorption may induce structural changes in the antigen that might increase its susceptibility to many host proteases involved in the generation of immune responses, favoring its presentation by professional antigen-presenting cells [34, 35].

Inorganic compounds used as adjuvants

Aluminum salts

Aluminum salts represent the most popular and ubiquitous class of vaccine adjuvant. Indeed, potassium aluminum sulphate ($KAl(SO_4)_2 \times 12\ H_2O$), whose characteristics are very similar to those of aluminum phosphate, was employed to obtain tetanus and

8. Gupta RK. Aluminium compounds as vaccine adjuvants. Adv Drug Deliv Rev. 1998;32: 155-72.

9. Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. Nat Rev Immunol. 2009;9:287-93.

10. Lahiri A, Das P, Chakravortty D. Engagement of TLR signaling as adjuvant: Towards smarter vaccine and beyond. Vaccine. 2008;26:6777-83.

11. Kawai T, Akira S. The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol. 2009;21(4):317-37.

12. Tagliabue A, Rappuoli R. Vaccine adjuvants: the dream becomes real. Hum Vaccine. 2008;4(5):347-9.

13. Lindblad EB. Aluminium adjuvants-in retrospect and prospect. Vaccine. 2004;22: 3658-68.

14. Glenny AT, Buttle GAH, Stevens MF. Rate of disappearance of diphtheria toxoid injected into rabbits and guinea-pigs: toxoid precipitated with alum. J Pathol. 1931;34:267-75.

15. Holt LB. Developments in diphtheria prophylaxis. London: William Heinemann, Ltd.; 1950.

16. Ramanathan VD, Badenoch-Jones P, Turk JL. Complement activation by aluminium and zirconium compounds. Immunology. 1979;37:881-8.

17. Harris J, Sharp FA, Lavelle Ed. C. The role of inflammasomes in the immunostimulatory effects of particulate vaccine adjuvants. Eur J Immunol. 2010;40:595-653.

18. Shar AF, Ruane D, Claass B, Creagh E, Harris J, *et al*. Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. Proc Natl Acad Sci USA. 2009;106(3):870-5.

19. Esch HH. Mechanisms of stimulation of the immune response by aluminum adjuvants. Vaccine. 2002;20:S34-S39.

20. Davenport FM, Hennessy AV, Askin FB. Lack of adjuvant effect of AlPO4 on purified influenza virus hemagglutinins in man. J Immunol. 1968;100(5):1139-40.

21. Cvjetanovic B, Uemura K. The present status of field and laboratory studies of typhoid and paratyphoid vaccines. Bull WHO. 1965;32:29-36.

22. Francis MJ, Fry CM, Rowlands DJ, Bittle JL, Houghten RA, Lerner RA, *et al*. Immune response to uncoupled peptides of foot-and-mouth disease virus. Immunology. 1987;61:1-6.

23. Bomford R. Aluminium salts: perspectives in their use as adjuvants. In: Gregoriadis G, Allison AC, Poste G, editors. Immunological adjuvants and vaccines. New York: Plenum Publishing Corp.; 1989. p. 35-41.

24. Francis MJ, Fry CM, Rowlands DJ, Brown F, Bittle JL, Houghten RA, *et al*. Immunological priming with synthetic peptides of foot-and-mouth disease virus. J Gen Virol. 1985;66:2347-54.

25. Lew AM, Anders RF, Edwards SJ, Langford CJ. Comparison of antibody avidity and titre elicited by peptide as a protein conjúgate or as expressed in vaccinia. Immunology. 1988;65(2):311-4.

diphtheria toxoid aluminates, thereby becoming the very first adjuvant to be used in human vaccines [36].

Aluminum hydroxide and phosphate, and later aluminum hydroxyphosphate sulfate, are the only aluminum-based adjuvants currently approved for manufacturing vaccines licensed for clinical use. The amount of aluminum per dose is restricted to no more than 0.85 mg in the USA, although European regulatory agencies have increased this limit to 1.25 mg since 1981. The table summarizes available vaccines currently licensed for use in humans that employ inorganic adjuvants [37].

Stanley Hem *et al.* studied the physico-chemical characteristics of aluminum hydroxide and phosphate, demonstrating that aluminum hydroxide, under the conditions employed for its application as adjuvant, is a crystalline solid [38-41]. Its structure corresponds to that of aluminum oxide hydroxide (AlO(OH)), a mineral known as boehmite that organizes into fibers with an average primary particle size of 4.5 × 2.2 × 10 nm. Thanks to this structural organization, aluminum hydroxide exhibits a large surface-mass ratio and a high capacity for adsorbing antigens. It has an isoelectric point that ranges from 9 to 11, and is marketed under the *Alhydrogel* trade name [38-41].

$AlPO_4$ is an amorphous, non-stoichiometric solid of variable OH-PO_4 ratio whose chemical formula is $Al(OH)_m(PO_4)_n$. It organizes into plates or sheets, and its average primary particle size is approximately 50 nm. Its isoelectric point ranges from 5 to 7, and is marketed under the *Adju-Phos* trade name [42].

In these complexes, the aquo ion Al^{3+} occupies tetrahedral and octahedral positions. The net surface charge of these two adjuvants impacts the way antigen adsorption takes place and the rate of antigen release after injection and exposure to the physiological medium, whose pH is close to neutral [41]. In solution, both adjuvants form porous aggregates with diameters ranging from 1 to 10 nm.

Aluminum hydroxide and aluminum phosphate exhibit different rates of solubilization, both *in vitro* and *in vivo*, into the interstitial fluid. While dissolution of the phosphate salt is relatively fast, the *in vitro* solubility of the hydroxide is poor, a finding that explains the prolonged persistence of this compound *in vivo*. The solubilization of these salts is favored in the presence of plasmatic, citric, lactic or malic acids, among others. According to the available data, 51 % of the injected aluminum phosphate is solubilized during the first month post-administration; a figure that drops to only 17 % in the case of aluminum hydroxide [42].

Amorphous hydroxyphosphate sulfate

Aluminum hydroxyphosphate sulfate ($Al_2(PO_4)$ (OH) (SO_4)) is a compound manufactured by Merck with a structure similar to that of aluminum phosphate, but clearly dissimilar properties. It is an amorphous lattice with an isoelectric point close to 7, which has been employed in a vaccine against Human Papillomavirus. The binding capacity of aluminum hydroxyphosphate sulfate for virus-like particles (VLP), and

26. Geerligs HJ, Weijer WJ, Welling GW, Welling-Wester S. The influence of different adjuvants on the immune response to a synthetic peptide comprising amino acid residues 9-21 of herpes simplex virus Type 1. J Immunol Methods. 1989;124(1):95-102.

27. Tripathy T, Ranjan D . Flocculation: A New Way to Treat the Waste Water. J Phys Sci. 2006;10:93-127.

28. World Health Organization. Temperature sensitivity of vaccines. WHO/IVB/06.10. Geneva: World Health Organization; 2006.

29. Rinella JV, White JL, Hem SL. Effect of pH on the Elution of Model Antigens from Aluminum-Containing Adjuvants. J Colloid Interface Sci. 1995;205(1):161-5.

30. Al-Shakhshir R, Regnier F, White JL, Hem SL. Effect of protein adsorption on the surface charge characteristics of aluminium-containing adjuvants. Vaccine. 1994;12(5):472-4.

31. Al-Shakhshir R, Lee AL, White JL, Hem SL. Interactions in Model Vaccines Composed of Mixtures of Aluminum-Containing Adjuvants. J Colloid Interface Sci. 1995;169:197-203.

32. Seeber SJ, White JL, Hem SL. Predicting the adsorption of proteins by aluminium-containing adjuvants. Vaccine. 1991;9(3): 201-3.

33. Clapp T, Siebert P, Chen D, Braun LJ. Vaccines with Aluminum-Containing Adjuvants: Optimizing Vaccine Efficacy and Thermal Stability. J Pharm Sci. 2011; 100(2):388-401.

Table. Vaccines based on inorganic adjuvants that are licensed for clinical use

Vaccine	Brand name	Adjuvant	Al^{3+} contents	Manufacturer
Antimeningococcal BC	VA-Mengoc-BC®	$Al(OH)_3$	2 mg	Finlay Institute, Cuba
Antipneumococcal	Prevenar®	$AlPO_4$	0.125 mg	Wyeth Pharmaceuticals Inc.
	Prevenar 13®	$AlPO_4$	0.125 mg	
Adsorbed anthrax	BioThrax®	$Al(OH)_3$	1.2 mg/mL	BioPort Corporation
DT	DT adsorbidos USP	Alum	≤ 0.25 mg	Sanofi Pasteur Inc.
	Decavac®		≤ 0.28 mg	
	Td (genérico)		≤ 0.28 mg	
DTaP	Boostrix®	Alum	≤ 0.39 mg	GlaxoSmithKline
	Infanrix®	$Al(OH)_3$	≤ 0.625 mg	
	Kinrix®*	$Al(OH)_3$	≤ 0.6 mg	
	Adacel®	$AlPO_4$	1.5 mg	Sanofi Pasteur Inc.
	Daptacel®	$AlPO_4$	0.33 mg	
	Tripedia®	Alum	≤ 0.17 mg	
DTaP and Hib	TriHIBit®	Alum	≤ 0.17 mg	Sanofi Pasteur Inc.
DTaP, hepatitis B, IPV	Pediarix®	$AlPO_4$ and $Al(OH)_3$	0.85 mg	GlaxoSmithKline
DTaP, IPV, Hib	Pentacel®	$AlPO_4$	0.33 mg	Sanofi Pasteur Inc.
Inactivated Hepatitis A	Havrix®	$Al(OH)_3$	0.5 mg/mL	GlaxoSmithKline
	Vaqta®	$Al(OH)_3$	0.45 mg/mL	Merck & Co., Inc.
Hepatitis B	Engerix-B®	$Al(OH)_3$	≤ 0.5 mg/mL	GlaxoSmithKline
	Recombivax HB®	$Al(OH)_3$	≤ 0.5 mg/mL	Merck & Co., Inc.
Hepatitis A and B	Twinrix®	$AlPO_4$ and $Al(OH)_3$	0.45 mg/mL	GlaxoSmithKline
Hib	PedvaxHIB®	$Al_2(PO_4)$ (OH) (SO_4)	0.22 mg	Merck & Co., Inc.
Hib and hepatitis B	Comvax®	Amorphous $Al(OH)_3$	0.225 mg	Merck & Co., Inc.
HPV	Cervarix®	$Al(OH)_3$	0.5 mg	GlaxoSmithKline
	Gardasil®	$Al_2(PO_4)$ (OH) (SO_4)	0.22 mg	Merck & Co., Inc.
HPV, tetravalent	Gardasil®	$Al_2(PO_4)$ (OH) (SO_4)	0.225 mg	Merck & Co., Inc.
HPV2, bivalent	Cervarix®	AS04 ($Al(OH)_3$)	0.5 mg	GlaxoSmithKline
Hepatitis B	Fendrix®	AS04 ($Al(OH)_3$)	0.5 mg	GlaxoSmithKline
Adsorbed	IPAD-T	$Ca_3(PO_4)_2$	1-3 mg/mL	Pasteur Institute, France

DT: diphtheria and tetanus toxoids; DTaP: diphtheria toxoid, tetanus toxoid and acellular pertussis; Hib: *Haemophilus influenzae*, type B; HPV: human papilloma virus; IPV: inactivated polio vaccine; TT: tetanus toxoid
* Kinrix® also includes IPV.

their subsequent stabilization, is larger than that of other aluminum salts [43].

Aluminum chloride

Aluminum chloride is an amphoteric compound that readily dissolves into water, forming aluminum hydroxide. It was used as an adjuvant for the production of antisera for cobra venom [44, 45].

Aluminum hydroxycarbonate

Aluminum hydroxycarbonate (Alum HC) forms through the pairing of a carbonate anion to an aluminum cation in an aluminum hydroxide gel. This compound has been used extensively as an antacid. A comparison of the adjuvant effect of aluminum hydroxycarbonate against that of 24 different adjuvants for eliciting a humoral immune response against Human Immunodeficiency Virus type 2 (HIV-2) in mice demonstrated that this compound, as all other aluminum-based adjuvants, is able to potentiate the induction of an antibody response against the gp120 antigen [46].

Aluminum silicate

In nature, aluminum silicates (Al_2SiO_5) are found only as hydrates. They have found extensive application in the pharmaceutical industry and in dentistry. A 1 % gel of bentonite, a form of aluminum silicate, has been the preferred formulation in vaccine adjuvant studies describing this compound. A study examining its potency for the induction of IgE responses in experimental animals and the production of IgG1 antibody found that it exhibited a behavior similar to that of alum [47]. Bentonite has also been used at 2 % in a study comparing the potency of four adjuvants for raising anti-rabies antibodies in horses [48].

A more recent study in sheep demonstrated that a vaccine against enterotoxaemia adsorbed in bentonite was a more economical alternative to the vaccine adsorbed into alum, with no loss of vaccine efficacy [49].

Manufacturing methods for aluminum salts

Aluminum oxides and hydroxides are generally prepared through the addition of NaOH or NH_3 solutions to a solution containing an aluminum salt ($AlCl_3$, $KAl(SO_4)_2 \times 12H_2O$). Other aluminum-based adjuvant salts are prepared similarly, the only difference being the simultaneous addition of a phosphate (Na_2HPO_4, NaH_2PO_4), bicarbonate or sulfate salt. The properties of the resulting gel (*i.e.*, surface area and charge, chemical composition, structure) are significantly influenced by the details of the manufacturing process, including factors, such as pH, temperature, concentration and chemical engineering variables (reactor geometry and rate of mixing). Quality specifications for aluminum-based gels used as adjuvants usually list parameters such as the contents of aluminum and some other ions (nitrates, sodium, chlorides, and sulfates), sterility, viscosity, sedimentation rate, adsorption capacity and isoelectric point, among others [50].

A number of amorphous hydrated structures, all in multiple equilibria with different aluminum species, are formed during the production of these gels. For instance, in aqueous solutions below pH 3, aluminum exists as a complex aquo ion $[Al(H_2O)_6]^{3+}$, usually written simply as Al^{3+}. At slightly higher pH the $[Al(H_2O)_6]^{3+}$ ion undergoes successive deprotonations, forming the $[Al(H_2O)_5(OH)]^{2+}$ ion and a number of hydrocomplexes [51, 52]. At pH close to 7, $Al(OH)_3$ begins to precipitate, and finally, at basic pH, $Al(OH)_3$ is solubilized again, forming the aluminate ion $[Al(OH)_4]$.

Calcium salts

Calcium phosphate

Calcium phosphate was originally examined as an alternative to aluminum-based adjuvants by Prof. Edgar H Relyveld, working at the Pasteur Institute. It has since been used as an adjuvant in vaccines against diphtheria, tetanus, whooping cough, poliomyelitis, tuberculosis, yellow fever, measles and hepatitis B, also forming part of immunotherapeutic preparations against a number of allergens [53-55].

Although the properties of calcium salts are similar to those of aluminum, the former exhibit a number of potential advantages. To begin with, calcium is a normal constituent of the human body and is, therefore, well tolerated; its capacity for adsorbing antigens is excellent, and antigen release proceeds only slowly; lastly, it stimulates the induction of IgG but not IgE antibodies, decreasing the possibility of long-term side effects that are usually detectable only in expensive large scale post-marketing trials. However, there have been sporadic cases of neurological reactions after the administration of *Bordetella pertussis* vaccines adsorbed with this adjuvant, and the World Health Organization, together with the European Pharmacopoeia, has therefore recommended an upper safety limit of 1.3 mg of calcium/dose [56].

The empirical formula of calcium phosphate as employed in adjuvants is approximated by $Ca_3(PO_4)_2$. X-ray diffraction, infrared spectroscopy and thermal analysis, among other techniques, have shown that commercially available calcium phosphate is a non-stoichiometric hydroxyapatite: $Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ (where x ranges from 0 to 2). Surface charge depends on pH, and its isoelectric point is 5.5. Calcium phosphate, therefore, is negatively charged at physiological pH, and adsorbs positively charged antigens through electrostatic interactions. This adjuvant can also adsorb phosphorylated antigens through ligand exchange via surface hydroxyls [57].

The adjuvant properties of calcium phosphate are strongly dependent on precipitation conditions, in a manner very similar to that described above for aluminum salts. Precipitates produced through the rapid mixing of two reactants adsorb 100 % of diphtheria toxoid, but those produced by slow addition only adsorb 58 % of the same dose; a phenomenon caused by molar Ca/P ratios that can vary from 1.35 to 1.83 depending on mixing rate [58].

Recently, calcium phosphate nanoparticles have been synthesized that show better physicochemical characteristics and performance than traditional preparations [59]. These nanoparticles, which produce less inflammatory reactions at the injection site, higher IgG2a titers and lower IgE titers than aluminum-based adjuvants, are regarded as a good alternative for immunization schemes involving viral antigens, and have already been applied in studies employing the

34. Peek JL, Russell Middaugh C, Berkland C. Nanotechnology in vaccine delivery. Adv Drug Deliv Rev. 2008;60:915-28.

35. Jones LS, Peek LJ, Power J, Markham A, Yazzie B, Middaugh R. Effects of Adsorption to Aluminium Salt Adjuvants on the Structure and Stability of Model Protein Antigens. J Biol Chem. 2005;280(14): 13406-14.

36. Lindblad EB. Aluminium compounds for use in vaccines. Immunol Cell Biol. 2004;82:497-505.

37. Baylor NW, Egan W, Richman P. Aluminum salts in vaccines-US perspective. Vaccine. 2002;20:S18-S23.

38. Clausi A, Cummiskey J, Merkley S, Carpenter J, Braun LJ, Randolph TW. Influence of particle size and antigen binding on effectiveness of aluminum salt adjuvants in a model lysozyme vaccine. J Pharm Sci. 2008;97(12):5252-62.

39. Shirodkar S, Hutchinson RL, Perry DL, White JL, Hem SL. Aluminum compounds used as adjuvants in vaccines. Pharm Res. 1990;7(12):1282-8.

40. Burrell LS, Johnston CT, Schulze D, Klein J, White JL, Hem SL. Aluminum phosphate adjuvants prepared by precipitation at constant pH. Part I: composition and structure. Vaccine. 2001;19:275-81.

41. Burrell LS, Johnston CT, Schulze D, Klein J, White JL, Hem SL. Aluminum phosphate adjuvants prepared by precipitation at constant pH. Part II: physicochemical properties. Vaccine. 2001;19:282-7.

42. Hem SL. Elimination of aluminum adjuvants. Vaccine. 2002;20:S40-S43.

43. Caulfield MJ, Shi L, Wang S, Wang B, Tobery TW, Henryck Mach, *et al*. Effect of Alternative Aluminum Adjuvants on the Absorption and Immunogenicity of HPV16 L1 VLPs in Mice. Hum Vaccines 2007;3(4):139-46.

44. Kawamura Y, Sawai Y. Study on Indian Cobra Venom toxoid. Snake. 1989; 21:6-8.

45. Kawamura Y, Sawai Y. Study on the immunogenicity of purified toxoid of Siamese Cobra (Naja Naja kaouthia) Venom. Snake. 1989;21:81-4.

46. Stieneker F, Kersten G, van Bloois L, Crommelin DJ, Hem SL, Löwer J, *et al*. Comparison of 24 different adjuvants for inactivated HIV-2 split whole virus as antigen in mice. Induction of titres of binding antibodies and toxicity of the formulations. Vaccine. 1995;13(1):45-53.

47. Fujimaki H, Ozawa M, Imai T, Kubota K, Watanabe N. Adjuvant effects of aluminum silicate on IgE and IgG1 antibody production in mice. Int Arch Allergy Appl Immunol. 1984;75(4):351-6.

48. Arora S, Sharma S, Goel SK, Singh US. Effect of different adjuvants in equines for the production of equine rabies immunoglobulin. Natl Med J India. 2005;18(6): 289-92.

49. Basavalingappa BS, Krishnamurthy GV, Suryanarayana VVS, Byregowda SM, Isloor S, Mayanna A, *et al*. Immune response of sheep to bentonite clay and alum adjuvanted enterotoxaemia vaccines. Indian J Animal Sci. 2008;78(4):339-41.

50. Matheis W, Zott A, Schwanig M. The role of the adsorption process for production and control combined adsorbed vaccines. Vaccine. 2002;20:67-73.

intranasal route to elicit mucosal immune responses in animal models [59-62]. Nano-structured calcium phosphate is, therefore, a good example of how it is possible to develop far superior adjuvants by simply modifying the size and morphology of existing compounds.

Calcium alginate

Calcium alginate is prepared from sodium alginate, which polymerizes in the presence of the former and forms depots where the antigen is adsorbed. The adjuvant effect of this preparation has been studied in mice, where it has been used in the production of antisera for *Bothrops asper* venom and in a number of assays involving antibacterial and antiviral vaccines. When used for experimental typhoid vaccines, it has been proven better at eliciting a humoral response than conventional adjuvants [63]. It should be noted, however, that calcium alginate is less potent as a vaccine adjuvant than Freund's, and has been shown to be unable to increase the immune response against diphtheria toxoid [64, 65].

Calcium chloride

Calcium chloride mixed with liposomes (forming cylindrically shaped cochleates) has been evaluated in intranasal vaccines used to stimulate mucosal immune response in animal models [66]. Also, it has recently been shown to have a good adjuvant effect when included in the AMVAD (*archaeal lipid mucosal vaccine adjuvant and delivery*) formulation, an intranasal formulation based on archaebacterial lipids that can promote a long-lasting systemic immune response in mice [67].

Magnesium compounds

Crystalline magnesium hydroxide has been employed in a mixture at equal proportions with amorphous aluminum hydroxycarbonate, sold under the registered trade mark of Imject Alum®. It has been shown to be an effective adjuvant in mice, but has not been examined yet in clinical trials [68, 69].

Another magnesium salt whose adjuvant effect has been studied is the penta-hydrate of magnesium hydroxycarbonate. In mice, the latter performed better than aluminum hydroxide for the induction of specific anti-TT antibodies [70].

Iron salts

Iron phosphate

Iron phosphate is an amorphous compound with an average particle size ranging from 0.01 to 300 µm. Studies in mice have shown that it is tolerated as well as aluminum hydroxide. Iron phosphate is an effective adjuvant for the induction of IgG1 antibodies, but is not better for this purpose than aluminum hydroxide (it is better, however, than iron hydroxide). The amount of iron per dose is limited by regulatory agencies to 0.2-1.4 mg per dose [71].

Colloidal iron

Colloidal iron is actually a mixture of iron (III) hydroxide and oxide, with varying stoichiometry and hydration levels. Its particle size ranges from 1 to 500 nm, and its adjuvant capacity is similar to that of aluminum hydroxide, but advantageously effective at stimulating the induction of cytotoxic T-lymphocytes. In experimental animals (mice), colloidal iron has been shown to increase the immunogenicity of TT and inactivated Tick-Borne Encephalitis Virus. Adsorption-wise, this type of colloid performs better than pure iron hydroxide gels, binding a wider range of proteins [72, 73].

Carbonyl iron

This compound forms spherical particles that have exhibited a potent adjuvant effect for the induction of allergic encephalomyelitis in rats. A similar adjuvant effect is exhibited by kaolin, but not other metallic iron suspensions such as iron oxide or iron-dextran. Carbonyl iron might become a useful tool for research into adjuvant mechanisms and cellular immunology due to its magnetic properties and ease of detection [74].

Compared to other alternatives, the iron-based adjuvants described above are handicapped by their smaller surface area/mass ratios, and therefore smaller adsorption capacities. In addition, these compounds exhibit slower absorption rates *in vivo*.

Zinc compounds

Zinc oxide

The adjuvant effect of zinc oxide has been studied in mice, where it stimulates Th2-type responses. According to the available data, zinc oxide is a safer alternative to aluminum hydroxide, as it is more potent, less toxic, and induces IgE in lower proportions [75, 76].

Zinc sulfate

Zinc sulfate has been used as adjuvant in formulations intended for topical use [77]. It was also studied in a clinical trial describing its effect on the immune response to a recombinant hepatitis B vaccine in the elderly, although this research has failed to detect a significant effect of this compound on antibody generation for this target population [78].

Zinc hydroxide

It is well known that zinc hydroxide is an effective protein and antigen sorbent. Not surprisingly, then, this compound has been shown to stimulate both cellular and humoral immune responses in mice and pigs. Adding lecithin increases the adjuvant effect and improves local tolerance for zinc hydroxide-based vaccines [78].

The adjuvanticity of zinc hydroxide in combination with calcium hydroxide, lecithin and hydrogenated poly-alfa-olefin is better than that of the individual components of this mixture.

Other studies have shown the effect of zinc chloride and acetate, with negative results. Not only their adjuvanticity is not detectable, but also local tolerance to their administration is low [78].

Manganese chloride

Manganese chloride has been tried in mice as an adjuvant, and found to stimulate the activity of natural killer cells (NK). It acts similarly to more complex compounds that induce the synthesis of interferons [79-81].

51. De Oliveira EC, Moita JM, Fujiwara FY. Aluminum Polyphosphate Thermoreversible Gels: A Study by 31P and 27Al NMR Spectroscopy. J Colloid Interface Sci. 1995;176(2):388-96.

52. Teagarden DL, Kozlowski JF, White JL, Hem SL. Aluminum chlorohydrate I: Structure studies. J Pharm Sci. 1981;70(7): 758-61.

53. Coursaget P, Yvonnet B, Relyveld EH, Barres JL, Diop-Mar I, Chiron PJ. Simultaneous administration of diphtheria-tetanus-pertussis-polio and hepatitis B vaccines in a simplified immunisalion programme: immune response to diphtheria toxoid, tetanus toxoid, pertussis and hepatitis B surface antigens. Infect Immun. 1986;51(3)784-7.

54. Relyveld EH. A history of toxoids. In: Plotkin SA, Fantini B, editors. Vaccinia, vaccination, vaccinology. New York: Elsevier; 1996. p. 95.

55. Relyveld EH, Hi-Nocq E, Raynaud M. Etude de la vaccination antidiphterique de sujets allergiques, avec une anatoxine pure adsorbee sur phosphate de calcium. Bull World Health Org. 1964;30:321-5.

56. Gupta RK, Siber GR. Comparison of adjuvant activities of aluminium phosphate, calcium phosphate and stearyl tyrosine for tetanus toxoid. Biologicals. 1994;22(1):53-63.

57. Jiang D, Premachandra GS, Johnston C, Hem SL. Structure and adsorption properties of commercial calcium phosphate adjuvant. Vaccine. 2004;23:693-8.

58. Relyveld EH. Preparation and use of calcium phosphate adsorbed vaccines. Dev Biol Stand. 1986;65:131-6.

59. He Q, Mitchell AR, Johnson SL, Wagner-Bartak C, Morcol T, Bell SJD. Calcium phosphate nanoparticle adjuvant. Clin Diagn Lab Immunol. 2000;7(6):899-903.

60. Abd el-Razek NEE, Shoman SA, Mohamed AF. Nanocapsulated Rift Valley Fever vaccine candidates and relative immunological and histopathological reactivity in out bred Swiss mice. J Vaccines Vaccin. 2011;2:115.

61. He Q, Mitchell A, Morcol T, Bell SJ. Calcium phosphate nanoparticles induce mucosal immunity and protection against herpes simplex virus type 2. Clin Diagn Lab Immunol. 2002;9(5):1021-4.

62. Contorni M, Singh M, Derek O'Hagan D, inventors; Novartis, assignee. Compositions with antigens adsorbed to calcium phosphate. United States patent US 20090035326. 2009 Feb 5.

63. Sterne M, Trim G. Enhancement of the potency of typhoid vaccines with calcium alginate. J Med Microbiol. 1970;3(4): 649-54.

64. Shapiro A, Modai Y, Kohn A. Efficacies of vaccines containing alginate adjuvant. J Appl Microbiol. 1967;30(2):304-11.

65. Kohn A, Helering I, Ben-Efraim S. Adjuvant properties of alginate in bacterial, viral and protein vaccines. Int Arch Allergy Appl Immunol. 1969;36(1-2):156-62.

66. Mannino RJ, Canki M, Feketeova E, Scolpino AJ, Wang Z, Zhang F, *et al*. Targeting immune response induction with cochleate and liposome-based vaccines. Adv Drug Deliv Rev. 1998;32(3):273-87.

Zirconium salts

Adjuvanticity studies have been performed for several zirconium salts and combinations thereof: zirconium and sodium lactate, zirconium lactate and aluminum, zirconium oxychloride, zirconium and aluminum glycinate and zirconium hydroxide. These compounds have been found to stimulate the immune response, producing larger titers of IgM antibodies in mice. Although the existing literature provides no further details, it is argued that these adjuvants induce a prolonged, more sustained production of antibodies.

In pigs, there is a direct relation between the extent of chronic inflammation induced by zirconium-based adjuvants (with the exception of zirconium hydroxide, ($Zr(OH)_4$) and activation of the complement cascade [16, 82].

Cerium nitrate

There are several different forms of cerium nitrate ($Ce(NO_3)_4$), although most medical applications of this salt employ its hexa-hydrate ($Ce(NO_3)_3 \times 6H_2O$). Currently, cerium nitrate is used as adjuvant in silver sulfadiazine creams. There is some data describing the in vitro evaluation of the protein adsorption capacity of cerium oxide and its uptake by lung adenocarcinoma cells [83].

Beryllium salts

A study in mice susceptible to infection by Leishmania spp. demonstrated that beryllium sulfate promoted significantly the production of interferon gamma (IFN-γ). This compound was shown to synergize with interleukin 12 (IL-12), stimulating the production of Th1 cytokines; these findings have led to the examination of the adjuvanticity of beryllium salts [84]. Beryllium oxide ($Be(OH)_2$) has been used as an adjuvant in animal experimentation [85].

Oxides

Aerosil

Aerosil 200 is a formulation based on highly dispersed 11 % (w/v) silicon dioxide that has found ample use as an auxiliary substance in pharmaceutical and cosmetic applications, due to its hydrophilicity. A similar formulation by the name of aerosil R972 is a hydrophobic dispersion of 1 % (w/v) silicon dioxide employed for the manufacture of pills from hygroscopic powders or granules [46]. The adjuvanticity of both formulations was evaluated in mice using inactivated HIV-2 antigens, finding that both were able to stimulate the production of antibodies against the gp120 glycoprotein.

Other oxides

The adjuvanticity of silica, (SiO_2), talcum powder ($Mg_3Si_4O_{10}(OH)_2$), aluminum oxide (Al_2O_3), tin oxide (SnO_2), zirconium oxide (ZrO_2), hematite (Fe_2O_3) and magnetite (Fe_3O_4) was evaluated in rats using ovalbumin as model antigen [86]. That study found that aluminum oxide, hematite and magnetite showed moderate adjuvanticity, but found tin and zirconium oxides to be very poor adjuvants. No link was detected between the hydrophobicity and hydrophilicity of these oxides and the magnitude of the resulting adjuvant effect.

Adjuvant mixtures

Algamulin

Taking advantage of the fact that gamma inulin, a natural algal polysaccharide, is a potent activator of the alternative complement pathway, Cooper *et al.* [87] combined aluminum hydroxide with gamma inulin. This combination, christened as 'algamulin', has been found to provide an adjuvant effect when combined with commercially available antigens such as diphtheria and tetanus toxoids, respiratory syncytial virus and protein E7 from the human papilloma virus [87].

AS04

AS04 (for Adjuvant system 04) is one of the very few examples of a novel, groundbreaking adjuvant that has actually been licensed for inclusion into prophylactic human vaccines. This compound is a combination of two different adjuvants with mutually complementary profiles: the time-tested aluminum hydroxide and monophosphoryl lipid A (MPL). This last is a purified, detoxified lipopolysaccharide from *Salmonella minnesota* that retains its affinity for Toll-like receptor 4 (TLR-4). AS04 is used in a commercially available Hepatitis B vaccine (marketed as Fendrix®) that is indicated preferentially for patients under hemodialysis and in immunosuppressive situations, and in a vaccine against the human papilloma virus (marketed as Cervarix®) [88].

Imject Alum®

Aluminum hydroxide-magnesium hydroxide, marketed under the Imject Alum® (Pierce Biotechnology, USA), is a 1:1 mixture of amorphous aluminum hydroxycarbonate with crystalline magnesium hydroxide that is very similar to Maalox®, a well-known antacid. This combination has only been used in studies involving experimental animals, but is reported as very effective. Its mechanism of action is based on the activation of inflammasome NLRP3 [89].

Noteworthy, adjuvant combinations represent a cost-efficient, quick solution whenever an adjuvant that can simultaneously stimulate a prolonged and intense antibody-based Th2 response and a potent Th1 response (fundamental for the protection against viruses and intracellular bacteria) is not available. Also, in addition to guaranteeing the intensity and quality of the induced response, these mixtures offer the possibility of including a homing molecule that may direct these responses to a specific immune response compartment.

Concluding remarks

Most inorganic adjuvants are employed as gels despite the previous availability of water-soluble divalent and trivalent metal salts of proven adjuvanticity, such as calcium gluconate, manganese gluconate, manganese glycerophosphate, aluminum acetate and aluminum salicylate [90]. The quality of these gels changes according to the manufacturer, hence, their adjuvant effect is sometimes far from optimum. This situation has pushed vaccine manufacturers into a search for alternatives of their own [91].

Most studies examining the adjuvanticity of inorganic compounds have used commercially available

67. Patel GB, Zhou H, Ponce A, Chen W. Mucosal and systemic immune responses by intranasal immunization using archaeal lipid-adjuvanted vaccines. Vaccine. 2007;25(51):8622-36.

68. Lambrecht BN, Kool M, Willart M, Hammad H. Mechanism of action of clinically approved adjuvants. Curr Opin Immunol. 2009;21:23-9.

69. Munks MW, McKee AS, Macleod MK, Powell RL, Degen JL, Reisdorph NA, *et al*. Aluminum adjuvants elicit fibrin-dependent extracellular traps *in vivo*. Blood. 2010;116(24):5191-9.

70. Kristensen N, Uldal J, Aasmul-Olsen S, Lund L, inventors; ALK-Abello A/S, assignee. Parenteral vaccine formulations and uses thereof. United States patent US 7785611 B2. 2010 Aug 31.

71. Sauzeat E, inventor; Sanofi Pasteur S.A., assignee. Vaccine composition comprising iron phosphate as vaccine adjuvant. United States patent US 06927235. 2005 Aug 9.

72. Eibl J, Leibl H, Mannhalter J, inventors; Tempo G, assignee. Adjuvant based on colloidal iron compounds. United States patent US 5895653. 1999 Apr 20.

73. Leibl H, Tomasits R, Brühl P, Kerschbaum A, Eibl MM, Mannhalter JW. Humoral and cellular immunity induced by antigens adjuvanted with colloidal iron hydroxide. Vaccine. 1999;17(9-10):1017-23.

74. Levine S, Sowinski R.Carbonyl iron: a new adjuvant for experimental autoimmune diseases. J Immunol. 1970;105(6): 1530-5.

75. Matsumura M, Nagata M, Nakamura K, Kawai M, Baba T, Yamaki K, *et al*. Adjuvant effect of cinc oxide on Th2 but not Th1 immune responses in mice. Immunopharmacol Immunotoxicol. 2010; 32(1):56-62.

76. Li T. Zinc hydroxide as a new vaccine adjuvant enhance the humoral immune response of HAV antigen [Master Dissertation]. Beijing: Peking Union Medical College; 2008.

77. Torvi J, Dambal SS, Indumati V. Effect of adjuvant oral zinc sulphate therapy in psoriasis patients. Int J Med Res. 2010;1(2): 106-10.

78. Dieter B, inventor; Behringwerke Aktiengesellschaft, assignee. Solutions containing antigen and cinc hydroxide or iron hydroxide as an adjuvant and processes for preparing such solutions. United States patent US 5252327. 1993 Oct 12.

79. Rogers RR, Garner RJ, Riddle MM, Luebke RW, Smialowicz RJ. Augmentation of murine natural killer cell activity by manganese chloride. Toxicol Appl Pharmacol. 1983;70(1):7-17.

80. Smialowicz RJ, Luebke RW, Rogers RR, Riddle MM, Rowe DG. Manganese chloride enhances natural cell-mediated immune effector cell function: Effects on macrophages. Immunopharmacology. 1985;9(1):1-11.

81. Smialowicz RJ, Rogers RR, Riddle MM, Luebke RW, Rowe DG, Garner RJ. Manganese chloride enhances murine cell-mediated cytotoxicity: effects on natural killer cells. J Immunopharmacol. 1984;6(1-2):1-23.

salts without paying due consideration to physicochemical properties such as polymorphisms, solubility constants, lattice energies, bonding (whether covalent or ionic), medium pH, oxidation status, particle size and antigen adsorption capacity, which impact directly the *depot* and *presentation* effects commonly attributed to adjuvants. Consequently, many research groups have rejected or ignored compounds that can potentially perform better as adjuvants than traditional aluminum salts [92].

Research in this field has focused not only on obtaining new adjuvants, but on developing new formulations from existing compounds. Nanotechnology offers the promise of inorganic salts whose properties can be shaped at a nanometric scale, potentially increasing their surface area and, thereby, favoring antigen absorption and intake by antigen-presenting cells (especially dendritic cells). The particulate nature of viruses and bacteria is mimicked much more efficiently by nanometer-sized aggregates, as exemplified by calcium phosphate and aluminum oxide nanoparticles.

Studying, obtaining and evaluating new polymorphic forms from safe and well-known aluminum and calcium compounds is an additional research avenue that will yield new structural, morphological and chemical data for re-evaluating existing adjuvants such as zinc and iron salts.

Currently, there is a trend towards the use of adjuvant mixtures, often combining compounds whose properties are mutually complementary as illustrated by the paradigmatic example of AS04. It should be stressed, however, that we are only scratching the surface of the world of potential applications offered by mixtures of inorganic adjuvants or the synthesis of artificial minerals with the exact chemical makeup required for increasing vaccine potency while keeping side effects to a minimum. A good starting point for the quest towards the ideal inorganic adjuvant would be the preparation of a compound mixing zinc hydroxide ($Zn(OH)_2$, a known Th1 promoter) with more traditional Th2-promoting calcium and aluminum salts.

In closing, it must be remarked that the application of inorganic compounds as vaccine adjuvants remains a lively and growing research field. The application of the latest advances in nanotechnology, together with the appearance of adjuvant combinations or the preparation of nanostructured compounds using salts or ions stimulating both the humoral and cellular arms of the immune system through different vaccination routes, will no doubt eventually yield compounds overcoming the limitations of current vaccine technology.

82. Shima S, Morita K, Tachikawa S, Ito T, Kurita H, Yoshida T, *et al*. IgM Antibody Production in Mice Intraperitoneally Injected with Zirconium Oxychloride. Br J Ind Med. 1987;44(9):633-7.

83. Patil S, Sandberg A, Heckert E, Self W, Seal S. Protein adsorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential. Biomaterials. 2007; 28(31):4600-7.

84. Lee JY, Atochina O, King B, Taylor L, Elloso M, Scott P, *et al*. Beryllium, an adjuvant that promotes gamma interferon production. Infect Immun. 2000;68(7):4032-9.

85. Hall JG. Studies on the adjuvant action of beryllitnn. IV. The preparation of beryllitnn containing macromolecules that induce immunoblast responses *in vivo*. Immunology. 1988;64:345-51.

86. Naim JO, van Oss CJ, Wu W, Giese RF, Nickerson PA. Mechanisms of adjuvancy: I-Metal oxides as adjuvants. Vaccine. 1997; 15(11):1183-93.

87. Cooper PD. Vaccine adjuvants based on gamma inulin. Pharm Biotechnol. 1995;6: 559-80.

88. Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H, *et al*. AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. J Immunol. 2009;183(10):6186-97.

89. Li H, Willingham SB, Ting JP, Re F. Cutting Edge: Inflammasome activation by Alum and Alum's adjuvant effect are mediated by NLRP3. J Immunol. 2008;181(1):17-21.

90. Aucouturier J, Ganne V, Trouve G, inventors; Societe d'Exploitation de Produits pour les Industries Chimiques, assignee. Vaccine composition of surfactants as adjuvant of immunity. United States patent US 7422748. 2008 Sep. 9.

91. Paneque A. El uso de las sales de aluminio como adyuvantes. IV International Symposium on Chemistry, 2010 Jun 1-4, Santa Clara, Cuba.

92. Paneque A. Compuestos inorgánicos como adyuvantes. 8th International Congress on Chemistry, Chemical Engineering and Biochemistry, 2012 Oct 9-12, La Habana, Cuba.

Received in June, 2012.
Accepted in April, 2013.