Immunology and Cell Biology (2004) **82**, 497–505 doi:10.1111/j.1440-1711.2004.01286.x

Special Feature

Aluminium compounds for use in vaccines

ERIK B LINDBLAD

Adjuvant Department, Brenntag Biosector, DK-3600 Frederikssund, Denmark

Summary Aluminium adjuvants are the most widely used adjuvants in both human and veterinary vaccines. These adjuvants have been used in practical vaccination for more than 60 years and are generally recognized as safe and as stimulators of Th2 immunity. The present review gives a short introduction to the pioneering research at the start of the use of aluminium compounds as adjuvants, including references on the chemistry of these compounds. Analytical methods for identifying the most commonly used aluminium compounds, such as boehmite and aluminium hydroxyphosphate, are mentioned. Emphasis is placed on the important factors for antigen adsorption and on the latest work using gene-deficient mice in the research of the mechanism of aluminium adjuvants in terms of cytokine and T-cell subset stimulation. Key references on the ability of aluminium adjuvants to stimulate IgE and also *in vivo* clearing of aluminium adjuvants are discussed. Furthermore, the review addresses the issue of local reactions in the context of injection route and local tissue disturbance. Possible new applications of aluminium adjuvants in, for example, combined aluminium-adsorbed protein and DNA oligonucleotide vaccines as well as the possible use of aluminium adjuvants in combination with IL-12 to stimulate Th1-type immune responses are mentioned.

Key words: aluminium, adjuvant, cytokine, mineral, vaccine.

Introduction

Aluminium adjuvants have been used in practical vaccination for more than half a century to induce early, high-titre, long-lasting protective immunity. Billions of doses of aluminium-adjuvanted vaccines have been administered over the years and they are, at present, the most widely used adjuvants in both veterinary and human vaccines. In general, aluminium adjuvants are regarded as safe when used in accordance with current vaccination schedules.[1,2]

In human vaccinations, aluminium adjuvants have primarily been used in tetanus, diphtheria, pertussis and poliomyelitis vaccines as part of standard child vaccination programmes in many countries for approximately 50 years. Aluminium adjuvants have also been introduced into hepatitis A and hepatitis B virus vaccines. Other aluminium-adsorbed vaccines against, for example, anthrax, are available for special risk groups. In veterinary medicine aluminium adjuvants have been used in a large number of vaccine formulations against viral[3–7] and bacterial[8–11] diseases, and in attempts to make antiparasite vaccines[12–15] (Table 1).

Glenny and coworkers were the first researchers to demonstrate the adjuvant effect of aluminium compounds in 1926. Glenny prepared a variety of diphtheria toxoid precipitates and investigated their immunogenicity. Among these were toxoids precipitated by the addition of potassium alum ($KAl(SO_4)_2{:}12H_2O$). Glenny observed that injecting the diphtheria toxoid as an alum precipitate led to a significant increase in the immune response against the toxoid.[16,17] Vaccines prepared in accordance with this principle have been used in practical vaccination and are referred to as alum-precipitated vaccines. Research showed that such preparations could be highly heterogenous, depending on which anions (such as bicarbonate, sulfate or phosphate) were present at the time of precipitation (e.g. as buffer constituents or growth media residues in the antigen solution).[18] However, preformed aluminium-hydroxide hydrated gels have the ability to adsorb protein antigens from an aqueous solution and these gels can be preformed in a well-defined and standardized way.[19] Vaccine preparations based on this approach are called aluminium-adsorbed vaccines in contrast to alum-precipitated vaccines. Aluminium phosphate was introduced as an adjuvant somewhat later. In 1946, Ericsson[20] devised a method in which diphtheria toxoid was coprecipitated into a matrix of aluminium phosphate. Holt,[21] shortly after, demonstrated that preformed aluminium phosphate, prepared from equimolar amounts of aluminium chloride and trisodium phosphate, acted as an adsorbant and was adjuvant active with diphtheria.

Data on the use of alum-precipitated vaccines can be found in older literature,[22] but in practical vaccinations the adsorption onto preformed hydroxide and aluminium phosphate gels has now almost completely substituted the alum precipitation in vaccine preparations. A number of other aluminium compounds have been investigated as adjuvants, for example, aluminium hydrochloride[23,24] and aluminium silicate,[25] but are not used in practical vaccinology. Occasionally the word 'alum' is used to describe both aluminium hydroxide and aluminium phosphate gels, but this is incorrect use of the terminology. Potassium alum, $KAl(SO_4)_2{:}12H_2O$, is in accordance with the chemical definition of an alum, whereas neither aluminium hydroxide nor aluminium phosphate are.

Correspondence: Dr EB Lindblad, Adjuvant Department, Brenntag Biosector, DK-3600 Frederikssund, Denmark.
Email: erik.lindblad@brenntag-nordic.com
Received 17 May 2004; accepted 5 July 2004.

Table 1 Examples of the application of aluminium adjuvants in veterinary vaccinology

Viral vaccines	Bacterial vaccines	Experimental anti-parasite vaccines
Avian infectious bronchitis virus	*Bacteroides nodosus*	*Cooperia punctata*
Canine hepatitis virus	*Bordetella bronchiseptica*	*Nematospiroides dubius*
Foot-and-mouth disease virus	*Clostridium botulinum*	*Onchocerca lienalis*
Newcastle disease virus	*Clostridium chauvoei*	*Trichinella spiralis*
	Clostridium novyi	
	Clostridium perfringens	
	Clostridium septicum	
	Clostridium sordellii	
	Haemophilus somnus	
	Leptospira interrogans	
	Pasteurella multocida	

Preparation and analysis of aluminium adjuvants

Aluminium hydroxide and aluminium phosphate adjuvants are generally prepared by exposing aqueous solutions of aluminium ions, typically as sulfates or chlorides, to alkaline conditions in a well-defined and controlled chemical environment. Various soluble aluminium salts can be used for the production of aluminium hydroxide, but the experimental conditions, temperature, concentration and even the rate of the addition of the reagents, strongly influence the results.[26,27] Anions present at the time of preparation may coprecipitate and change the characteristics away from those of a 'pure' aluminium hydroxide. Aluminium phosphate gel can be seen as an example of such a preparation, where the soluble aluminium salts are exposed to alkaline conditions in the presence of phosphate ions.

Stanley Hem's group at Purdue University has intensively studied the physicochemical nature of the aluminium hydroxide and aluminium phosphate gel preparations that have commonly been used as vaccine adjuvants for approximately 25 years. Using X-ray crystallography and infrared spectroscopy they demonstrated a boehmite-like (aluminium oxyhydroxide) pattern in preparations traditionally known as aluminium hydroxide, whereas commercialized aluminium phosphate gel adjuvant was identified as amorphous aluminium hydroxyphosphate.[28] They were able to calculate an average primary crystallite size of 4.5 × 2.2 × 10 nm for the boehmite preparations.[29]

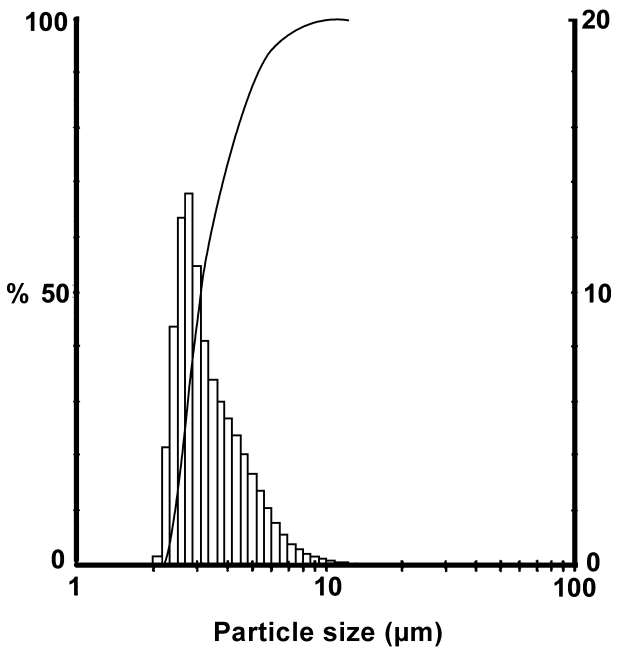

Figure 1 Typical particle size distribution of aluminium hydroxide adjuvant (Alhydrogel) measured using Malvern laser diffraction equipment.

Analytical methods

A number of physicochemical characteristics of the aluminium adjuvants can now be described using modern analytical methods.

Particle size distributions of the aluminium gel particles can be obtained using, for example, laser diffraction analysis. An example of this, obtained using Malvern equipment, is given in Fig. 1. The size of the aluminium hydroxide particles is comparable to the size of microorganisms and is adequate for uptake by phagocytosis by APC.

Stanley Hem's group has investigated the fine structure and crystallite size of aluminium adjuvants using X-ray diffraction and infrared spectroscopy. The X-ray diffraction band width measured at the half height at the 020 reflection (6.46Å), occasionally referred to as WHH_{020}, expresses the crystallite size.[28] Infrared spectroscopy applied to aluminium hydroxide preparations reveals the structure known as boehmite.[28]

The protein adsorption capacity of the adjuvant can be measured using a variety of analytical methods. In general, it is done by comparing the protein content in the aqueous phase of the antigen solution before and after adsorption onto the adjuvant. One of the classical methods, the Ramon flocculation test (named after the Frenchman Gaston Ramon who introduced the adjuvant concept around 1925), is still used to determine the adsorption of diphtheria and tetanus toxoid.[30] If an antibody specific for the antigen you wish to adsorb is available, adsorption can be measured using immunoprecipitation techniques, by using either quantitative immunoelectrophoresis[31] or single radial immunodiffusion.[32] Without the use of an antibody it can be tested spectrophotometrically (e.g. the BCA method).[33] However, it is important to note that contamination with the fine fraction of aluminium gel particles can disturb the spectrophotometrical readings by light-scatter effects. ELISA methods in which aluminium-adsorbed

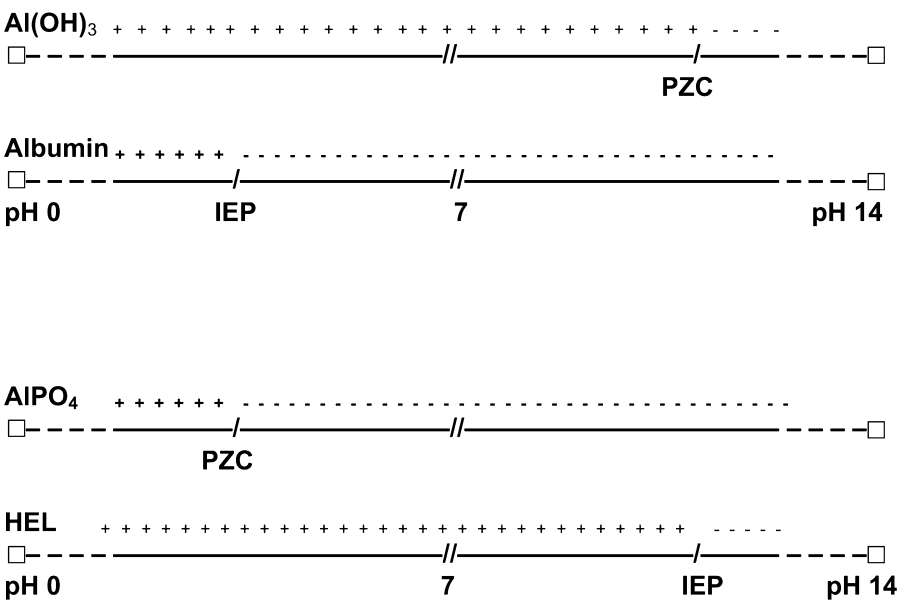

Figure 2 In the pH range between the isoelectric point (IEP) of the antigen and the point of zero charge (PZC) of the mineral adjuvant there is the basis for electrostatic attraction because of opposite charge. The alkaline PZC for $Al(OH)_3$ makes it suitable for adsorption of acidic IEP proteins as shown by albumin, whereas the acidic PZC of $AlPO_4$ makes it suitable for adsorption of alkaline IEP proteins as shown by hen egg lysozyme.

antigens can be used directly as the antigens in the ELISA have been designed.[34] ELISA methods can also be applied for *in vitro* assessment of various viral antigens, that is, pseudorabies, porcine parvovirus and infectious bovine rhinotracheitis vaccines adsorbed onto aluminium hydroxide adjuvant.[35]

Differential adsorption of complex mixtures of antigens can be measured using either immunoelectrophoresis or by HPLC. If an antiserum raised against the complex antigen mixture is available, a crossed 2-D immunoelectrophoresis will reveal if single components from the complex solution of proteins remain unadsorbed. To use this approach, the precipitation band pattern from an electrophoresis run on the complex antigen mixture prior to adsorption is compared with the bands of an electrophoresis run on the supernatant of the same mixture after adsorption. Unadsorbed components will retain their immunoprecipitation band pattern, whereas missing bands or a reduced height of the bands are indicative of complete or partial protein adsorption.[31] A comparison of HPLC chromatograms of the antigen-mixture liquid phase before and after adsorption may provide similar information.

The aluminium content in the final vaccine can be monitored using atomic adsorption spectrometry as devised by May *et al*.[36]

Mechanisms of adjuvant activity

The immunostimulating effect of the traditional aluminium adjuvants is highly complex and must be attributed to several different mechanisms. In the past,[17] the function of a repository adjuvant was attributed to delayed clearing from the injection site and sustaining a gradual release of adsorbed antigen from the inoculated depot. Although it quickly became obvious that the gradual release explanation was inadequate, the physical adsorption of antigen onto the adjuvant is still considered to be a very important mechanism for the function of these adjuvants. Over the past two decades the role of T cells and cytokine profiles has also been described.

Antigen adsorption

Although the literature provides examples of publications where injection of adjuvant and unadsorbed antigen at distant sites leads to immunostimulation towards the antigen,[37] this result is not consistent[38] and the nature of the antigen chosen in the research may provide part of the explanation for these differing conclusions. As a general rule the antigen should be adsorbed onto the adjuvant prior to immunization.

A soluble antigen may upon adsorption be presented to the immunocompetent cells in a 'particulate' manner that could facilitate antigen targeting (i.e. favour uptake by APC). It is likely that APC may be more efficient in antigen uptake by phagocytosis than by pinocytosis. There is experimental evidence of enhanced uptake of aluminium adsorbed tetanus toxoid when compared to the soluble toxoid by APC.[39]

The physicochemical mechanisms behind the antigen adsorption itself are complex and depend on the nature of the individual antigen. Some mechanisms may predominate over others.

As a general guideline, for many protein antigens adsorption is best accomplished in the pH interval between the isoelectric point (IEP) of the protein antigen and the point of zero charge (PZC) of the aluminium adjuvant. This applies to both aluminium hydroxide and aluminium phosphate adjuvants. In this interval the adjuvant and the antigen will have opposite electrical charges, facilitating electrostatic attraction and adsorption (Fig. 2). Seeber *et al*.[40] concluded that aluminium hydroxide should be superior to aluminium phosphate in adsorbing proteins with an acid IEP and vice versa for proteins with an alkaline IEP.

However, if the antigen contains phosphorylated groups ligand exchange between the antigen-associated phosphate and hydroxyl groups of the adjuvant may account for a high-affinity binding to the adjuvant. This is the case with HBsAg particles[41] and has been shown in experiments using phosphorylated alpha-casein as the model antigen.[42]

The primary mechanisms responsible for adsorption have been explained, in part, by electrostatic attraction and, in part,

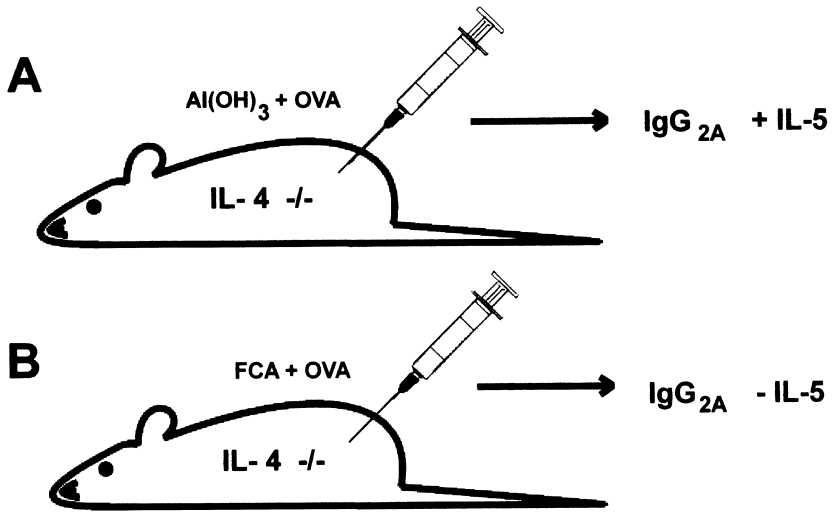

Figure 3 Immunization with OVA + $Al(OH)_3$ in IL-4-gene-disrupted mice gave rise to stimulation of the Th1-associated antibody subclass, IgG2a, plus IL-5, which is normally considered a Th2-associated cytokine. In contrast, stimulation of IL-5 was not observed after immunization with OVA + FCA in IL-4-gene-disrupted mice (see Brewer *et al*.[56]).

by anionic ligand exchange.[43] In addition, other intermolecular binding forces, including hydrophilic–hydrophobic interaction, may play a role in protein adsorption[44] and each binding force may play it's role in a given antigen–adjuvant combination depending on the nature of the antigen and the chemical environment (pH, ionic strength, presence of surfactants etc.).[45–47]

T-cell reactivity, antibody subclasses and stimulation of cytokines

In the past it was often claimed that aluminium adjuvants were not 'T-cell adjuvants'. But this conclusion was generally based on work in which a 'T-cell adjuvant' was primarily considered an adjuvant capable of inducing a delayed-type hypersensitivity (DTH) response. It is correct that aluminium adjuvants are not efficient DTH inducers in rodents.[48] There is also very little evidence that aluminium adjuvants should be able to generate MHC class I restricted cytotoxic T cells; to date only a single report from Dillon *et al*.[49] using a recombinant influenza vaccine in mice. However, as early as the late 1970s the ability of aluminium adjuvants to induce eosinophilia was shown to require the presence of T cells[50] and the reaction profile of aluminium adjuvants is now known to include stimulation of $CD4^+$ T cells.[51]

Studies examining the impact of interleukins in model systems were initiated almost 20 years ago and in recent times more studies have been conducted because of the introduction of gene-disrupted mice. Among the early observations in classical animal models was the demonstration that aluminium-adsorbed tetanus toxoid led to an increase in antigen-induced T-cell proliferation, apparently resulting from the increased release of IL-1.[39] In contrast, IL-1 does not appear to be important in the augmentation of the primary antibody response in rabbits immunized with aluminium adjuvant.[52]

Grun and Maurer[51] demonstrated that anti-IL-1 or anti-IL-4 were able to inhibit an antigen-specific T-cell proliferative response after immunization with aluminium adjuvant, but not if the mice were immunized with Freund's Complete Adjuvant. However, the proliferative responses were inhibited by anti-CD4 antibody, regardless of the adjuvant used. This indicated that the proliferating $CD4^+$ T cells from mice immunized using aluminium adjuvant were from the Th2 subset. Lindblad and coworkers[53] found a corresponding profile in C57BL/6J mice when they examined IgG2a and IgG1 subclass assays and reverse transcription–polymerase chain reaction (RT-PCR) for IL-4- and IL-10-specific mRNA in the regional draining lymph nodes at day 7 following vaccination with aluminium-adjuvanted vaccine.

Although it is now generally accepted that aluminium adjuvants are Th2 stimulators, it is interesting to note that a complex between $Al(OH)_3$ and IL-12 ($Al(OH)_3$/IL-12) induced a Th1 response, rather than a Th2 reponse, when used as an adjuvant[54] and the Th1-promoting effect of the $Al(OH)_3$/IL-12 complex was greatly augmented by the coadministration of exogenous IL-18.[55]

A new line of research has been initiated with the introduction of gene-disrupted mice, which has facilitated studies examining the significance of interleukins.

In IL-4-gene-disrupted mice immunization with OVA + $Al(OH)_3$ gave IgG2a titres of a similar magnitude to OVA + FCA.[56] Interestingly, the group immunized with OVA + $Al(OH)_3$ continued to produce IL-5 (a cytokine normally associated with the Th2 profile). In contrast, when the $IL\text{-}4^{-/-}$ mice had been immunized with FCA, a similar stimulation of IL-5 was not observed (Fig. 3). These results support the idea that the major role of aluminium-induced IL-4 in Th-subset stimulation is to downregulate the Th1 response.

The role of IL-18 in the adjuvant mechanisms of aluminium hydroxide and its effect on Th2 induction has been demonstrated.[55] Brewer *et al.* revealed that IL-18-deficient mice immunized with OVA + $Al(OH)_3$ had reduced IL-4 production in lymph node cells compared to wild-type mice. However, if they added exogenous IL-18 the aluminium-induced Th2 response was not further enhanced. Although the aluminium adjuvant led to reduced IL-4 production in $IL\text{-}18^{-/-}$ mice, this was not accompanied by a reduced level of serum

anti-OVA IgG1. Apparently, there is a poor correlation between this particular antibody subclass and IL-4 production.

Stimulation of IgE

It is well established that aluminium adjuvants stimulate the production of IgE as part of the overall Th2 profile.[57,58] Although this has been considered a disadvantage, it has been difficult to demonstrate cases where vaccination with aluminium adjuvants has led to IgE-mediated allergy towards the vaccine antigen in practical vaccination. In contrast, aluminium adjuvants have been used to hyposensitize allergic patients for many years with good results.

There are some interesting similarities between the immune response (e.g. stimulation of IgE and eosinophilia) elicited by some helminthic parasites and the immune response following immunization with aluminium adjuvants that make these adjuvants interesting candidates for antiparasitic vaccines. A good vaccine against, for example, schistosomiasis would be of major importance in the health of people in developing countries. To date, however, only partial protection has been achieved in animal models.[59,60] In a special mouse hybrid (C57BL/6J × DBA/2) F1, which is known to be a high-producer of IgE, it was possible to induce a significant degree of protection against schistosomiasis.[60] The protected mice had high titres of specific IgE antibody after immunization using sonicated parasite antigens and aluminium hydroxide adjuvant. Mice immunized with sonicated antigen and FCA had low-level protection and lower IgE titres. Although the group immunized with FCA had high titres of specific total Ig,[60] protection against schistosomiasis was still lower. This suggests a protective superiority of specific IgE against helminthic infections.

A very useful way to examine IgE/Th2 stimulation in rodent models is to immunize groups of animals using aluminium adjuvant versus Freund's Complete Adjuvant, respectively, and assess the resulting antibody and cytokine profiles. Uede *et al*.[61,62] pioneered this line of research over two decades ago using keyhole limpet haemocyanin as the antigen. Their study revealed the involvement of glycosylation-enhancing factors and Fcγ R+ T cells in a dichotomous regulatory pathway in which aluminium adjuvant stimulated the synthesis of IgE and FCA supressed it.[61,62]

The introduction of gene-disrupted mice has led to more sophisticated applications of the aluminium versus FCA model for studying IgE regulation. Brewer and coworkers used the same approach to compare the adjuvant profiles of aluminium hydroxide versus FCA.[56] They found that there was no IgE production in IL-4-gene-disrupted mice (IL-4$^{-/-}$) regardless of whether aluminium adjuvant or FCA was used as the adjuvant. This suggests that IL-4 is an essential prerequisite for the induction of IgE by aluminium adjuvants.

Dose–response considerations

The optimum dose of adjuvant is normally determined empirically in a pilot trial, but helpful guidelines are available in the literature. In veterinary vaccines there is no defined maximum limit for the allowed content of aluminium adjuvants and, in general, the dose is set with the aim of balancing efficacy and local reactogenicity. However, there are limitations for the content of aluminium allowed in vaccines for humans. These limits are 1.25 mg aluminium per dose in Europe,[63] and in USA 0.85 mg aluminium per dose if determined by assay, 1.14 mg if determined by calculation and 1.25 mg if safety and efficacy data justifies it.[64]

For the dose–response relationship of aluminium adjuvants in combination with bacterial antigens the immunomodulation observed may reflect a composite effect between the aluminium adjuvant and immunomodulatory and adjuvant active bacterial substances, such as LPS, trehalose dimycolate ('cord factor'), muramyl peptides from peptidoglycans or CpG motifs from bacterial DNA.[65]

Limitations to the applicability of aluminium adjuvants

One obvious limitation in the application of aluminium adjuvants lies in the clear Th2 profile of these adjuvants. A Th2-biased immune response is not likely to protect against diseases for which Th1 immunity and MHC class I restricted CTL are essential for protection (e.g. intracellular parasites or tuberculosis).[53] Another limitation lies in the fact that traditional aluminium-adsorbed vaccines are frost sensitive and thus not lyophilizable.

Aluminium adjuvants have failed to provide satisfactory augmentation of the immune response in a number of cases, including influenza and typhoid fever vaccines.[66,67] In some recent approaches to vaccine preparation, aluminium adjuvants have shown limitations in their applicability in vaccines based on small-size peptides.[68] In some cases, for example, with foot-and-mouth disease virus peptides, the problem could be overcome by conjugating the peptide to a larger carrier molecule,[69] while in others it could not.[70,71] Aluminium adjuvants have been tested in a few DNA vaccine formulations. In these cases it was revealed that aluminium hydroxide had an inhibiting effect, whereas aluminium phosphate adjuvant augmented the immune response against the antigen encoded by the nucleotide.[72,73] The high content of phosphate in the DNA molecule apparently gives a high binding affinity of the nucleotide to the aluminium hydroxide, which in turn prevents the RNA from getting access to and then translating the nucleotides into protein.[73]

In vivo clearing of aluminium adjuvants

Mammalian organisms are constantly being exposed to aluminium compounds in the environment. As a consequence aluminium is found in the blood and serum of humans and animals whether or not they have been vaccinated using aluminium adjuvants. The major source of this aluminium is apparently oral intake with food and drinking water. Martyn *et al*.,[74] based on a study in Britain, calculated the average daily intake of aluminium by humans to be 5–10 mg. Individuals with normal renal functions would excrete this aluminium uptake with the urine. In contrast to other metallic ions, such as Zn^{++} and Mg^{++}, aluminium apparently does not act as an essential trace element or coenzyme in normal metabolism. The clearing *in vivo* of aluminium adjuvants has been investigated in rabbits using adjuvants prepared from the isotope ^{26}Al.[75] Blood and urine excreted ^{26}Al was followed using accelerator mass spectroscopy for a period of 28 days. As early as 1 h after i.m. injection radioactive-labelled Al could

be detected in the blood and approximately threefold more ^{26}Al was excreted from animals vaccinated with aluminium phosphate than those vaccinated with aluminium hydroxide. Thus, it appears that interstitial fluid containing organic acids with an α-hydroxy carboxylic acid, which is able to chelate Al, react more readily with aluminium phosphate than with aluminium hydroxide.[75] It is of interest that following injection of adjuvant containing 0.85 mg Al the normal plasma concentration of Al in rabbits (30 ng Al ml^{-1}) only rose approximately 2 ng Al ml^{-1} during the experiment. Flarend[75] calculated that a similar Al dose injected into humans, provided similar clearing kinetics existed, would lead to an estimated increase of serum Al of only 0.04 ng Al ml^{-1}, which is equal to 0.8% above the normal level of approximately 5 ng Al ml^{-1}. Based on these figures it appears unlikely that the amount of aluminium administered via vaccination would contribute significantly to the general exposure of humans to aluminium or to the serum levels of aluminium.

Side-effects

Aluminium hydroxide and aluminium phosphate adjuvants have been used for more than half a century now and are generally regarded as safe when used according to current immunization schedules.[2] In 1993 the United States the National Cooperative Vaccine Development Group on safety evaluation of vaccine adjuvants with the participation of FDA representatives concluded that 'the extensive experience with this class of adjuvant for vaccine use has indicated that it is safe'.[76]

There is no evidence that aluminium adjuvants themselves should be immunogenic and act as haptens, accordingly they are not likely to cause harmful immune complex reactions and observations of contact hypersensitivity reactions are rare.[2,77] The aluminium adjuvants are not in themselves pyrogenic and there is no evidence of carcinogenicity or teratogenicity attributed to their use. In contrast, aluminium hydroxide has a significant binding affinity to endotoxins that may reduce the reactogenicity of some adsorbed vaccines containing, for example, residual LPS.

Cases of local reactions have occasionally been reported.[78] These may comprise swellings, indurations, erythemas and cutaneous nodules that can persist for up to 8 weeks and occasionally longer.[79] These reports often describe cases of allergic patients undergoing hyposensitizing therapy, during which they receive a large number of injections of adsorbed allergenic extracts over a limited period of time. The aluminium adjuvants should be regarded as depot-forming or repository adjuvants. With these adjuvants the formation of an inflammatory focus attracting immunocompetent cells shortly after injection must be expected.[1] After injection macrophages are attracted to the site to phagocytize and clear the inoculum. The local reaction may be negligible if the inoculum is rapidly dispersed from the injection site. However, if the inoculum resides for a prolonged period of time at the injection site (as is the case with repository adjuvants such as mineral adjuvants or water-in-oil emulsions) then *in situ* accumulation of phagocytic and immunocompetent cells may in some cases manifest itself as an inflammatory focus accompanied by a transient swelling, local irritation and redness. Some observations of aluminium-adsorbed vaccines giving rise to more local reactions than unadsorbed vaccines with plain toxoid[80] could, in part, be explained by the plain toxoid vaccine being dispersed from the injection site before a local reaction was established.

According to the *danger model* described by Matzinger[81] it is conceivable that such an inflammatory focus, through disturbance of local tissue integrity, elicits danger signals and generates heat-shock proteins and pro-inflammatory cytokines. Such molecules may contribute to stimulation of the primary immune response against the antigen.

Any visible or palpable reaction at the injection site is in principle *non grata* because it hinders the obtaining of a hypothetical 'ideal adjuvant'. However, it is important to realize that the mechanisms described are part of a normal functioning immune system. Hence, it may not be achievable to use repository adjuvants without temporarily inducing an inflammatory focus around the inoculum.

Vaccinations may be given subcutaneously or intramuscularly and the injection modus is not without importance with respect to local reactogenicity. When immunizing using the subcutaneous route the vaccine inoculum is introduced into a compartment with numerous sensory neurones (in contrast to the intramuscular compartment). In this case the introduction of a local inflammatory response may more easily give rise to irritation and itching reactions. In addition, a transient swelling, as a consequence of the inflammatory focus formed, may be more easily palpable through the skin. When immunizing using the intramuscular route a similar size swelling may be less easily visible and palpable because it is located in deeper lying tissue.

Whether or not adsorption onto aluminium adjuvants leads to increased or decreased vaccine reactogenicity is not clear.[80,82] However, Butler *et al*. found that adsorption onto aluminium hydroxide (Alhydrogel) significantly reduced the side-effects with combined diptheria–tetanus–pertussis vaccines.[83] The binding affinity of LPS to aluminium hydroxide is well established and is much higher than the binding to aluminium phosphate,[84] probably because of the phosphate content of LPS. It is conceivable that acute toxicity is reduced in adsorbed vaccines simply because of a delayed release of toxic vaccine constituents, such as pertussis toxin, peptidoglycans from Gram-negative cell walls or LPS from the injection site. Norimatsu showed that adsorption of LPS onto aluminium hydroxide prior to injection inhibited or mitigated systemic effects including trembling, transient leucopenia and elevated serum TNF that would otherwise be observed following i.m. injection of LPS in saline.[85] In addition, the level of IL-6 after administration of LPS was reduced if the LPS was adsorbed to aluminium hydroxide prior to injection.[84]

A number of observations of side-effects following vaccination with adjuvanted vaccines must, however, be attributed to the vaccine preservatives (e.g. thiomersal, betapropriolactone or formaldehyde) or to bacterial toxins from the antigen preparation.[86]

Epilogue

Aluminium adjuvants have beyond doubt proven their efficiency in a large number of applications as vaccine adjuvants in both human and veterinary immunoprofylaxis with very few problems. In addition, they are relatively inexpensive,

which may not be relevant from a scientific point of view, but may be of great importance in establishing effective vaccination programmes in developing countries where funding is limited.

When evaluating the profile of an adjuvant for possible new applications very few adjuvants can match the extremely comprehensive cohorts that are available for aluminium adjuvants with respect to records of efficacy and safety profiles, which after more than half a century of use reach the lifespan of humans.

There is still a lot of research to be done to get a clearer picture of the mode of action of aluminium adjuvants. Incubation of dendritic cells (DC) with aluminium adjuvant failed to activate the DC as judged by increased expression of MHC class II and costimulatory molecules. This indicates that aluminium adjuvants, in contrast to bacteria-derived adjuvants (e.g. LPS, CpG), are not directly addressing toll-like receptors.[87] But, it would be an interesting working hypothesis if heat-shock proteins and pro-inflammatory cytokines could indeed be demonstrated to be a consequence of the local tissue disturbance created by the injected aluminium-adsorbed vaccine. As heat-shock proteins contain highly conserved molecular regions with structural similarities to bacterial components it is possible that interaction with toll-like receptors could take place *in vivo* as a secondary reaction derived from the local tissue disturbance.

Aluminium adjuvants have their limitations, especially with regard to their profound Th2 reactivity, which means that there are vaccines in which they will have little or no effect. However, in the future, modified formulations with a more balanced Th1–Th2 profile may become available for use in practical vaccinology along with more potent Th1 stimulators, such as monophosphoryl lipid A. The potential application of the $Al(OH)_3$/IL-12 complex[54,55] for use in vaccines for Th1 stimulation is yet to be explored in detail. The same is the case for another composite formulation, Algammulin (gamma inulin + aluminium hydroxide), which has been shown to give a more balanced immune response with stimulation of IgG2a isotypes in mice indicative of a mixed Th1–Th2 stimulation.[88] It is also interesting to note that it appears to be possible to combine traditional aluminium-phosphate-adsorbed protein vaccines with DNA oligonucleotide vaccines resulting in a combination of the two different technologies in a single vaccine formulation.[73] The potential of these as well as other approaches to expand the use of aluminium adjuvants into new vaccine applications requires further research.

References

1 World Health Organization. *Immunological Adjuvants. Technical Report Series 595.* Geneva: World Health Organization, 1976.

2 Edelman R. Vaccine Adjuvants. *Rev. Infect. Dis.* 1980; **2**: 370–83.

3 McDougall JS. Avian Infectious Bronchitis: The Protection Afforded by an Inactivated Virus Vaccine. *Vet. Rec.* 1969; **85**: 378–80.

4 Wilson JHG, Hermann-Dekkers WM, Leemans-Dessy S, de Meijer JW. Experiments with an inactivated hepatitis leptospirosis vaccine in vaccination programmes for dogs. *Vet. Rec.* 1977; **100**: 552–4.

5 Sellers RF, Herniman KAJ. Early Protection of Pigs Against Foot-and-Mouth Disease. *Br. Vet. J.* 1974; **130**: 440–5.

6 Hyslop NStG, Morrow AW. The Influence of Aluminium Hydroxide Content, Dose Volume and the Inclusion of Saponin on the Efficacy of Inactivated Foot-and-Mouth Disease Vaccines. *Res. Vet. Sci.* 1969; **10**: 109–20.

7 Pini A, Danskin D, Coackley W. Comparative Evaluation of the Potency of Beta-propiolactone Inactivated Newcastle Disease Vaccines Prepared from a Lentogenic and a Velogenic Strain. *Vet. Rec.* 1965; **77**: 127–9.

8 Thorley CM, Egerton JR. Comparison of Alum-Adsorbed or Non-Alum-Adsorbed Oil Emulsion Vaccines Containing, either Pilate or Non-Pilate *Bacteroides nodosus* Cells in Inducing and Maintaining Resistance of Sheep to Experimental Foot Rot. *Res. Vet. Sci.* 1981; **30**: 32–7.

9 McCandlish IAP, Thompson H, Wright NG. Vaccination Against Canine Bordetellosis using an Aluminium Hydroxide Adjuvant Vaccine. *Res. Vet. Sci.* 1978; **25**: 51–7.

10 Nagy LK, Penn CW. Protective Antigens in Bovine Pasteurellosis. *Dev. Biol. Stand.* 1974; **26**: 65–76.

11 Ris DR, Hamel KL. *Leptospira interrogans serovar: pomona* Vaccines with Different Adjuvants in Cattle. *NZ Vet. J.* 1979; **27**: 169–71.

12 Leland SE, Sofield WL, Minocha HC. Immunogenic Effects of Culture-Derived Exoantigens of *Cooperia punctata* on Calves Before and After Challenge Exposure with Infective Larvae. *Am. J. Vet. Res.* 1988; **49**: 366–79.

13 Monroy FG, Adams JH, Dobson C, Bast IJ. *Nematospiroides dubius*: Influence of Adjuvants on Immunity in Mice Vaccinated with Antigens Isolated by Affinity Chromatography from Adult Worms. *Exp. Parasitol.* 1989; **68**: 67–73.

14 Carlow CKS, Bianco AE. Resistance of *Onchocerca lienalis* Microfilariae in Mice Conferred by Egg Antigens of Homologous and Heterologous *Onchocerca* Species. *Parasitology* 1987; **94**: 485–96.

15 Gamble HR, Murrell KD, Marti HP. Inoculation of Pigs Against *Trichinella spiralis* using Larval Excretory-Secretory Antigens. *Am. J. Vet. Res.* 1986; **47**: 2396–9.

16 Glenny AT, Pope CG, Waddington H, Wallace U. Immunological Notes XVII to XXIV. *J. Patho1.* 1926; **29**: 31–40.

17 Glenny AT, Buttle GAH, Stevens MF. Rate of Disappearance of Diphtheria Toxoid Injected into Rabbits and Guinea-Pigs: Toxoid Precipitated with Alum. *J. Patho1.* 1931; **34**: 267–75.

18 Holt LB. *Developments in Diphtheria Prophylaxis.* London: Heinemann Ltd, 1950.

19 Maschmann E, Küster E, Fischer W. Über die Fähigkeit des Tonerde-Präparates B, Diphtherie-Toxin zu adsorbieren. *Ber. Dtsch. Chem. Ges.* 1931; **64**: 2174–8.

20 Ericsson H. Purification and adsorption of diphtheria toxoid. *Nature* 1946; **158**: 350–1.

21 Holt LB. Purified precipitated diphtheria toxoid of constant composition. *Lancet* 1947; **8**: 282–5.

22 Volk VK, Bunney WE. Diphtheria immunization with fluid toxoid and alum-precipitated toxoid. *Am. J. Public Health* 1942; **32**: 690–9.

23 Kawamura Y, Sawai Y. Study on Indian Cobra Venom Toxoid. *Snake* 1989; **21**: 6–8.

24 Kawamura Y, Sawai Y. Study on the Immunogenicity of Purified Toxoid of Siamese Cobra *(Naja Naja kaouthia)* Venom. *Snake* 1989; **21**: 81–4.

25 Fujimaki H, Ozawa M, Imai T, Kubota K, Watanabe N. Adjuvant Effects of Aluminium Silicate on IgE and IgG1 Antibody Production in Mice. *Int. Archs Allergy Appl. Immun.* 1984; **75**: 351–6.

26 Willstätter R, Kraut H. Über ein Tonerde-Gel von der Formel $Al(OH)_3$. II. Mitteilung über Hydrate und Hydrogele. *Ber. Dtsch. Chem. Ges.* 1923; **56**: 1117–21.

27 Willstätter R, Kraut H. Über die Hydroxide und ihre Hydrate in den verschiedenen Tonerde-Gelen. V. Mitteilung über Hydrate und Hydrogele. *Ber. Dtsch. Chem. Ges.* 1924; **57**: 1082–91.

28 Shirodkar S, Hutchinson RL, Perry DL, White JL, Hem SL. Aluminium Compounds Used as Adjuvants in Vaccines. *Pharm. Res.* 1990; **7**: 1282–8.

29 Johnston CF, Wang S-L, Hem SL. Measuring the surface area of aluminium hydroxide adjuvant. *J. Pharm. Sci.* 2002; **91**: 1702–6.

30 Relyveld EH, Raynaud M. Methods of Toxin and Antitoxin Assays *in vitro*. In: Williams CA, Chase MW (eds). *Methods in Immunology and Immunochemistry*, Vol. IV. New York: Academic Press, 1977; 283–93.

31 Weeke B, Weeke E, Løwenstein H. The Adsorption of Serum Proteins to Aluminium Hydroxide Gel Examined by Means of Quantitative Immunoelectrophoresis. In: Axelsen NH (eds). *Quantitative Immunoelectrophoresis. New developments and applications.* Oslo: Universitetsforlaget, 1975; 149–54.

32 Lindblad EB. Aluminium Adjuvants. In: Stewart-Tull DES (ed.). *The Theory and Practical Application of Adjuvants*. New York: John Wiley & Sons, 1995; 21–35.

33 Smith PK, Krohn RI, Hermanson GT *et al.* Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 1985; **150**: 76–85.

34 Thiele GM, Rogers J, Collins M, Yasuda N, Smith D, McDonald TL. An Enzyme-Linked Immunosorbent Assay for the Detection of Antitetanus Toxoid Antibody Using Aluminium-Adsorbed Coating Antigen. *J. Clin. Lab. Anal.* 1990; **4**: 126–9.

35 Katz JB, Hanson SK, Patterson PA, Stoll IR. *In vitro* assessment of viral antigen content in inactivated aluminium hydroxide adjuvanted vaccines. *J. Virol. Meth.* 1989; **25**: 101–8.

36 May JC, Progar JJ, Chin R. The aluminium content of biological products containing aluminium adjuvants: determination by atomic absorption spectrometry. *J. Biol. Stand.* 1984; **12**: 175–83.

37 Flebbe LM, Braley-Mullen H. Immunopotentiating effects of the adjuvants SGP and Quil-A. I. Antibody responses to T-dependent and T-independent antigens. *Cell. Immunol.* 1986; **99**: 119–27.

38 Gupta RK, Rost BE, Relyveld E, Siber GR. Adjuvant Properties of Aluminum and Calcium Compounds. In: Powell MF, Newman MJ (eds). *Vaccine Design: The Subunit, Adjuvant Approach.* New York: Plenum Press, 1995; 229–48.

39 Mannhalter JW, Neychev HO, Zlabinger GJ, Ahmad R, Eibl MM. Modulation of the human immune response by the non-toxic and non-pyrogenic adjuvant aluminium hydroxide: Effect on antigen uptake and antigen presentation. *Clin. Exp. Immunol.* 1985; **61**: 143–51.

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

41 Iyer S, Robinett RSR, HogenEsch H, Hem SL. Mechanism of adsorption of hepatitis B surface antigen by aluminium hydroxide adjuvant. *Vaccine* 2004; **22**: 1475–9.

42 Iyer S, HogenEsch H, Hem SL. Effect of the degree of phosphate substitution in aluminium hydroxide adjuvant on the adsorption of phosphorylated proteins. *Pharm. Dev. Tech.* 2003; **8**: 81–6.

43 Hem SL, White JL. Characterization of Aluminium Hydroxide for Use as an Adjuvant in Parenteral Vaccines. *J. Parent. Sci. Tech.* 1984; **38**: 2–11.

44 Al-Shakhshir RH, Regnier FE, White JL, Hem SL. Contribution of electrostatic and hydrophobic interactions to the adsorption of proteins by aluminium-containing adjuvants. *Vaccine* 1995; **13**: 41–4.

45 Rinella JV, White JL, Hem SL. Effect of anions on model aluminium-adjuvant-containing vaccines. *J. Colloid Interface Sci.* 1995; **172**: 121–30.

46 Rinella JV, White JL, Hem SL. Effect of pH on the elution of model antigens from aluminium-containing adjuvants. *J. Colloid Interface Sci.* 1998; **205**: 161–5.

47 Rinella JV, Workman RF, Hermodson MA, White JL, Hem SL. Elutability of proteins from aluminium-containing vaccine adjuvants by treatment with surfactants. *J. Colloid Interface Sci.* 1998; **197**: 48–56.

48 Bomford R. The comparative selectivity of adjuvants for humoral and cell-mediated immunity. II. Effect on delayed-type hypersensitivity in the mouse and guinea pig, and cell-mediated immunity to tumour antigens in the mouse of Freund's incomplete and complete adjuvants, Alhydrogel, *Corynebacterium parvum, Bordetella pertussis,* muramyl dipeptide and saponin. *Clin. Exp. Immunol.* 1980; **39**: 435–41.

49 Dillon SB, Demuth SG, Schneider MA *et al.* Induction of protective class I MHC-restricted CTL in mice by a recombinant influenza vaccine in aluminium hydroxide adjuvant. *Vaccine* 1992; **10**: 309–18.

50 Walls RS. Eosinophil Response to Alum Adjuvants. Involvement of T Cells in Non-Antigen-Dependent Mechanisms. *Proc. Soc. Exp. Biol. Med.* 1977; **156**: 431–5.

51 Grun JL, Maurer PH. Different T Helper Cell Subsets Elicited in Mice Utilizing Two Different Adjuvant Vehicles. The Role of Endogenous Interleukin-1 in Proliferative Responses. *Cell. Immunol.* 1989; **121**: 134–45.

52 Sagara T, Mori S, Ohkawara S, Goto F, Takagi K, Yoshinaga M. A limited role of IL-1 in immune-enhancement by adjuvants. *Immunol.* 1990; **71**: 251–7.

53 Lindblad EB, Elhay MJ, Silva R, Appelberg R, Andersen P. Adjuvant modulation of immune responses to tuberculosis subunit vaccines. *Infect. Immun.* 1997; **65**: 623–9.

54 Jankovic D, Caspar P, Zweig M *et al.* Adsorption to aluminium hydroxide promotes the activity of IL-12 as an adjuvant for antibody as well as type 1 cytokine responses to HIV-1 gp 120. *J. Immunol.* 1999; **163**: 4481–8.

55 Pollock KGJ, Conacher M, Wei X-Q, Alexander J, Brewer JM. Interleukin-18 plays a role in both the alum-induced T helper 2 response and the T helper 1 response induced by alum-absorbed interleukin-12. *Immunology* 2003; **108**: 137–43.

56 Brewer JM, Conacher M, Satoskar A, Bluethmann H, Alexander J. In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to Freund's complete adjuvant, but continues to induce T helper 2 cytokine production. *Eur. J. Immunol.* 1996; **26**: 2062–6.

57 Kenney JS, Hughes BW, Masada MP, Allison AC. Influence of adjuvants on the quantity, affinity, isotype and epitope specificity of murine antibodies. *J. Immunol. Meth.* 1989; **121**: 157–66.

58 Hamaoka T, Katz DH, Bloch KJ, Benacerraf B. Hapten-Specific IgE Antibody Responses in Mice. I. Secondary IgE Response in Irradiated Recipients of Syngeneic Primed Spleen Cells. *J. Exp. Med.* 1973; **138**: 306–11.

59 Knopf PM, Goldberg M, Grossi CA, Cappello M, Coulter ST. Induction of Resistance to *Schistosoma mansoni* by Immunization with Subfractions of Worms. *Am. J. Trop. Med. Hyg.* 1988; **38**: 515–28.

60 Horowitz S, Smolarsky M, Arnon R. Protection against *Schistosoma mansoni* achieved by immunization with sonicated parasite. *Eur. J. Immunol.* 1982; **12**: 327–32.

61 Uede T, Huff TF, Ishizaka K. Formation of IgE Binding Factors by Rat T Lymphocytes. V. Effect of Adjuvant for the Priming Immunization on the Nature of IgE Binding Factors Formed by Antigenic Stimulation. *J. Immunol.* 1982; **129**: 1384–90.

62 Uede T, Ishizaka K. Formation of IgE Binding Factors by Rat T Lymphocytes. VI. Cellular Mechanisms for the Formation of IgE-Potentiating Factor and IgE-Suppressive Factor by Antigenic Stimulation of Antigen Primed Spleen Cells. *J. Immunol.* 1982; **129**: 1391–7.

63 European Pharmacopeia. *Vaccines for Human Use*, 3rd edn. 1997; 1697.

64 Code of Federal Regulations 21, vol. 7: Sec. 610.15 (Constituent Materials). Revised 1 April 2003. US Government Publication.

65 Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 2002; **20**: 709–60.

66 Davenport FM, Hennessy AV, Askin FB. Lack of Adjuvant Effect of $AlPO_4$ on Purified Influenza Virus Hemagglutinins in Man. *J. Immunol.* 1968; **100**: 1139–40.

67 Cvjetanovic B, Uemura K. The Present Status of Field and Laboratory Studies of Typhoid and Paratyphoid Vaccines. *Bull. WHO* 1965; **32**: 29–36.

68 Francis MJ, Fry CM, Rowlands DJ *et al.* Immune response to uncoupled peptides of foot-and-mouth disease virus. *Immunology* 1987; **61**: 1–6.

69 Francis MJ, Fry CM, Rowlands DJ *et al.* Immunological Priming with Synthetic Peptides of Foot-and-Mouth Disease Virus. *J. General Vir.* 1985; **66**: 2347–54.

70 Lew AM, Anders RF, Edwards SJ, Langford CJ. Comparison of Antibody Avidity and Titre Elicited by Peptide as a Protein Conjugate or as Expressed in Vaccinia. *Immunology* 1988; **65**: 311–14.

71 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. Meth.* 1989; **124**: 95–102.

72 Ulmer JB, DeWitt CM, Chastain M *et al.* Enhancement of DNA vaccine potency using conventional aluminum adjuvants. *Vaccine* 2000; **18**: 18–28.

73 Kwissa M, Lindblad EB, Schirmbeck R, Reimann J. Co-delivery of a DNA vaccine and a protein vaccine with aluminium phosphate stimulates a potent and multivalent immune response. *J. Mol. Med.* 2003; **81**: 502–10.

74 Martyn CN, Barker DJP, Osmond C, Harris EC, Edwardson JA, Lacey RF. Geographical relation between Alzheimers disease and aluminium in drinking water. *Lancet* 1989; **1**: 59–62.

75 Flarend RE, Hem SL, White JL *et al. In vivo* absorption of aluminium containing vaccines using ^{26}Al. *Vaccine* 1997; **15**: 1314–18.

76 Goldenthal KL, Cavagnaro JA, Alving C, Vogel FR. Safety Evaluation of Vaccine Adjuvants. NCVDG Working Groups. *AIDS Res. Human Retroviruses* 1993; **9** (Suppl. 1): s47–s51.

77 Böhler-Sommeregger K, Lindemayr H. Contact sensitivity to aluminium. *Contact Dermatitis* 1986; **15**: 278–81.

78 Gupta RK, Relyveld EH, Lindblad EB, Bizzini B, Ben-Efraim S, Gupta CK. Adjuvants — a balance between toxicity and adjuvanticity. *Vaccine* 1993; **11**: 293–306.

79 Frost L, Johansen P, Pedersen S, Veien N, Østergaard P, Nielsen M. Persistent Subcutaneous Nodules in Children Hyposensitized with Aluminium-Containing Allergen Extracts. *Allergy* 1985; **40**: 368–72.

80 Collier LH, Polakoff S, Mortimer J. Reactions and Antibody Responses to Reinforcing Doses of Adsorbed and Plain Tetanus Vaccines. *Lancet* 1979; **1**(8131): 1364–7.

81 Matzinger P. Tolerance, danger and the extended family. *Ann. Rev. Immunol.* 1994; **12**: 991–1045.

82 Aprile MA, Wardlaw AC. Aluminium Coumpounds as Adjuvants for Vaccines and Toxoids in Man: a Review. *Can. J. Public Health* 1966; **57**: 343–54.

83 Butler NR, Voyce MA, Burland WL, Hilton ML. Advantages of Aluminium Hydroxide Adsorbed Combined Diphtheria, Tetanus, and Pertussis Vaccines for the Immunization of Infants. *Br. Med. J.* 1969; **1**: 663–6.

84 Shi Y, HogenEsch H, Regnier FE, Hem SL. Detoxification of endotoxin by aluminium hydroxide adjuvant. *Vaccine* 2001; **19**: 1747–52.

85 Norimatsu M, Ogikubo Y, Aoki A *et al.* Effects of aluminium adjuvant on systemic reactions of lipopolysaccharides in swine. *Vaccine* 1995; **13**: 1325–9.

86 Chaby R, Caroff M. Lipopolysaccharides of *Bordetella pertussis* Endotoxin. In: Wardlaw AC, Parton P (eds). *Pathogenesis and Immunity in Pertussis.* New York: John Wiley and Sons, 1988; 247–71.

87 Sun H, Pollock KGJ, Brewer JM. Analysis of the role of vaccine adjuvants in modulating dendritic cell activation and antigen presentation *in vitro*. *Vaccine* 2003; **21**: 849–55.

88 Cooper PD, McComb C, Steele EJ. The adjuvanticity of Algammulin, a new vaccine adjuvant. *Vaccine* 1991; **9**: 408–15.