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Review

(How) do aluminium adjuvants work?

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Abstract

The aluminium compounds, originally identified as adjuvants over 70 years ago, remain unique in their widespread application to human vaccines. Given this history, it is surprising that the physicochemical interactions between aluminium compounds and antigens are relatively poorly understood. This has clearly been a contributing factor to vaccine failures, for example, through inappropriate selection of aluminium species or buffers. Similarly, the mechanism(s) of action of aluminium adjuvants are relatively unstudied, although it appears that these agents fail to fit within the current principles underlying activation of the immune response. This review aims to examine recent developments in our understanding of the physicochemical and biological aspects of research into aluminium adjuvants.
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Keywords: Aluminium adjuvant; Vaccine; Dendritic cells

Contents

1. Introduction 10
2. Aluminium adjuvants 11
3. Adjuvant-induced immune activation 12
4. Direct activation of APCs by aluminium adjuvants 12
5. Indirect activation of APCs by aluminium adjuvants 13
6. Conclusions 14
References 14

1. Introduction

According to recent WHO statistics, HIV, malaria and tuberculosis are responsible for 4.9, 2.7 and 2.2%, respectively, of annual global mortality [1]. Chemotherapy for these diseases is expensive and typically, of long duration, resulting in reduced patient compliance, low effectiveness and increased potential for multi-drug resistance. Clearly, an effective vaccine against any of these diseases would make a considerable contribution to world health. Traditionally, the development of vaccines has focused on the identification of antigens that elicit the appropriate, specific immune response to generate immunity. With increasing progress in vaccine research, this process has become increasingly refined, moving from whole organisms to extracts, toxoids or single proteins isolated from pathogens or expressed by recombinant DNA techniques. With progress in immunology, in particular our understanding of T and B lymphocyte antigen receptors and their ligands, this research has moved towards conjugate and even peptide vaccines. The underlying objective in this increasing refinement has been the promise

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of vaccines with increased effectiveness, improved safety profiles and greater ease of manufacture. However, one unwanted side effect of this process has been the concomitant reduction in immunogenicity. In experimental situations, this lack of immunogenicity could be easily remedied by formulation of the antigen with a vaccine adjuvant. Adjuvants have been defined as "agents that act non-specifically to increase the specific immune response or responses to an antigen" [2]. Essentially this means that adjuvants can act to increase the specific immune response to an administered antigen and should also act with a wide range of antigens. Janeway also called adjuvants 'the immunologist's dirty little secret' in cognisance of the fact that while a great deal of effort has gone into the design of vaccine antigens, the study of adjuvants has been largely empirical and the mechanisms of how they exert their activity are far less studied or understood [3]. One of the confounding issues in investigating the mechanisms of action of vaccine adjuvants has been the diverse range of unrelated substances that have been shown to have adjuvant activity [4]. These include oil emulsions, natural and synthetic surfactants, mineral gels, bacterial derivatives and esoteric substances, such as breadcrumbs and tapioca [5–7]. Trying to devise a unifying theory to explain this phenomenon is clearly not a straightforward task.

2. Aluminium adjuvants

The aluminium compounds were originally identified as adjuvants over 70 years ago [8] and they remain unique in their widespread application to human vaccines. A variety of chemical forms of aluminium compound have been employed, since the use of potassium alum-precipitated proteins by Glenny et al. The main aim of these alternatives has been to produce more homogenous vaccine preparations, as precipitation with potassium alum is highly susceptible to the interfering effects of charged ions. This is a significant issue as it is generally accepted that physical association of antigen and aluminium is required for adjuvant activity, indeed the WHO recommends that at least 80% of diphtheria and tetanus toxoids be adsorbed to aluminium adjuvant in these vaccines [9]. Current vaccines therefore employ preformed aluminium hydroxide or aluminium phosphate gels, often, though incorrectly, referred to as 'alum'. The application of these adjuvants has largely been limited to certain bacterial and viral vaccines where protection against infection is known to be dependent on the generation of neutralising antibodies [10,11]. The apparently simple systematic names of the aluminium adjuvants belie a complex, heterogeneous physical structure, comprising amorphous crystalline aluminium oxyhydroxide or aluminium oxyphosphate [12]. Furthermore, the process of adsorption of protein solutions to aluminium adjuvants is a complicated process that is only recently being fully understood [13,14]. It appears that proteins bind to aluminium adjuvants in two basic ways. Firstly, and probably most commonly, proteins can adsorb by electrostatic interactions between proteins and the positively charged aluminium hydroxide [14]. However, this presumes the pH of the buffer used lies above the isoelectric point of the protein, which while common for serum proteins, for example, is in no way universal. This is particularly true of shorter proteins or peptides that can quite often have high isoelectric point due to a preponderance of basic amino acid residues. Despite this, negatively charged aluminium phosphate gels can also bind proteins that should be repelled by their net negative charges. This second method of binding involves 'ligand exchange' between hydroxyl and phosphate groups, for example, the hydroxyl groups of proteins and aluminium phosphates, although it has also been demonstrated to occur between aluminium hydroxide and proteins containing phosphate groups [13,14]. While the use of preformed aluminium adjuvants has reduced vaccine heterogeneity, clearly factors, such as pH, buffer strength, ionic components and physical features of the protein remain to affect the reproducibility of these adjuvants. These variables have clearly contributed to the failure of aluminium adjuvants in a number of vaccines [11,15].

A further significant problem facing aluminium adjuvants is, however, the restricted range of immune responses induced by this adjuvant. Early studies demonstrated that while aluminium adjuvants are very effective in promoting the expansion of humoral immune responses including IgE production [16], they fail to stimulate cell mediated immune (CMI) responses, such as delayed type hypersensitivity (DTH) [17]. Subsequent studies have demonstrated that while aluminium adjuvants can stimulate Th2 type responses and the production of cytokines, such as IL-4 and IL-5, as well as B cell production of IgG1 and IgE, it fails to stimulate Th1 responses, such as IFNγ production and B cell IgG2a secretion [5,18]. This poses a significant problem to the continued development of vaccines, particularly given that the effectiveness of vaccines against the three diseases causing most global mortality, HIV, tuberculosis and malaria, are entirely or partially dependent on the generation of Th1 type immunity [19–22]. In experimental situations, this problem can be overcome by employing one of the wide range of experimental adjuvants, in particular Freund's complete adjuvant (FCA; [23]), that are known to induce antigen specific Th1 responses [4]. However, the use of FCA causes inflammation, in-duration or necrosis with disseminated granulomas being reported in the lungs, liver, kidneys, heart, lymph nodes and skeletal muscles of rabbits or rats after subcutaneous or intravenous injection [24]. Clearly, the lack of a clinically applicable adjuvant that induces Th1 responses poses a significant obstacle to the effective clinical application of vaccines against these diseases. Many developmental adjuvants have aimed to fulfil this role, yet none of them has succeeded. One reason underlying this lack of success is our poor integration of adjuvant research with our current understanding of immune response induction. Clearly, understanding these mechanisms would

facilitate the rational development of new and more effective adjuvants.

3. Adjuvant-induced immune activation

Induction of antigen specific responses to protein antigens requires the activation of T helper cells. The signalling requirements for this activation have been described based on two distinct signals that are generated in the T cell and induced by APCs. Signal 1 is the cognate signal delivered to T cells by peptide/MHC class II complexes on the surface of APCs [25]. As mentioned above, signal 1 has occupied a great deal of vaccine development activity. However, signal 1 alone is usually inadequate for effective vaccination without the presence of a second signal, signal 2 [25,26]. A number of studies have demonstrated that signal 2 is induced in T cells by co-stimulatory molecules or cytokines expressed by APCs and is essential for T cell activation leading to immunity. In the absence of effective provision of signal 2, T cells enter a state of functional hyporesponsiveness or anergy [26]. In terms of vaccine development, it appears that both whole organisms and adjuvants can program APCs to express costimulatory molecules to induce signal 2 in T cells [27]. Although this model may explain the lack of immunogenicity of peptide vaccines, it fails to explain how vaccine adjuvants or whole organisms can actually program the ability to induce signal 2.

A number of in vitro and in vivo studies have demonstrated that dendritic cells (DCs) are the most effective APC population in inducing activation and proliferation of naïve T cells. DCs are strategically situated in diverse tissues, such as the epidermis and gastrointestinal mucosa, where the potential for invasion by pathogens is high [28,29], however, for optimal T cell activation, these cells must be activated in order to firstly migrate to T cell areas of lymph nodes, and secondly to acquire increased antigen presentation and costimulatory activity [28,29]. A set of evolutionarily conserved motifs expressed by bacteria and viruses are recognised by members of a series of mammalian receptors, called toll-like receptors (TLRs) [3,30,31]. These highly conserved receptors are expressed on macrophages and B cells, as well as DCs and they have been shown to mediate APC activation on encountering the appropriate microbial stimulation. Significantly, microbial agents with the capacity to activate DCs also possess adjuvant activity [32,33], and it is generally accepted that the function of adjuvants is mediated through DC activation. Clearly, these studies have been performed to determine how the immune system responds to invasion by pathogens, where an evolutionary pressure exists to allow the immune system to recognise infectious non-self. In contrast, the broad spectrum of vaccine adjuvants, ranging from synthetic particles to plant glycosides to inorganic materials, such as the aluminium adjuvants, lacks any such organising pressure and strains the concept that non-self recognition is entirely due to a limited set of germ-line encoded receptors [34].

4. Direct activation of APCs by aluminium adjuvants

Early studies suggested that aluminium adjuvants had two direct effects on antigen presenting cells, enhanced uptake of antigen and increased IL-1 production that may explain enhanced antigen-specific T cell responses [35]. More recently, studies with murine bone marrow-derived macrophages demonstrated that, as with other particulate, non-microbial adjuvants, aluminium adjuvants fail to induce costimulatory molecule expression on DCs following in vitro incubation. However, while particulates, such as liposomes (NISV) and PLGA microparticles could increase the presentation of entrapped, though not unentrapped antigens, aluminium adjuvants could not [36]. The requirement for association of antigen with particulate adjuvants is well recognised, and in the past, it has been proposed that the ability of particulates to target antigen to phagocytes may underlie their adjuvant activity. Furthermore, the mechanism of antigen internalisation can determine the efficiency of antigen processing and presentation to T cells by phagocytes. In vitro studies show that antigen prepared in particles with mean sizes of greater than 200 nm are targeted for phagocytosis by bone marrow-derived macrophages and produce quantitatively more efficient presentation than fluid phase uptake of similar quantities of antigen prepared in smaller particles (<200 nm) [37]. Similarly, it has also been shown that the ability of aluminium adjuvants to increase internalisation of antigen is dependent on association of antigen with aluminium adjuvants particles and presumably phagocytic uptake [13]. Significantly, the ability of aluminium adjuvants to adsorb antigens is critically dependent on the environmental milieu that the vaccine is delivered into with protein rich solutions, such as tissue culture medium or interstitial fluid being capable of displacing antigens previously adsorbed to aluminium adjuvants. This is particularly true of antigens that bind to aluminium adjuvants through electrostatic interactions rather than ligand exchange, for example, ovalbumin [13]. Therefore, another reason that aluminium adjuvants may fail is through lack of antigen association with adjuvant not only during formulation, but also following administration, and consequently a loss of antigen targeting to phagocytes or uptake via phagocytosis.

As mentioned above, the inability of non-microbial or sterile adjuvants, such as aluminium to directly up-regulate costimulatory molecule expression on dendritic cells in vitro makes sense from an evolutionary perspective. However, some studies do report APC activation in response to these adjuvants. Ulanova et al. first reported that human PBMCs exposed to proprietary aluminium hydroxide (Alhydrogel), with a low endotoxin level (<1 pg/ml) were partially activated, upregulating expression of MHC class II and costimulatory molecules, downregulating CD14 and acquiring the appearance of dendritic cells. The authors also demonstrated that the activation observed could be inhibited by IL-4 neutralisation and suggest that T cells in the PBMC population may be the source of this cytokine [38]. In contrast to

this study, Rimaniol et al. demonstrated that PBMC-derived macrophages could also be activated by aluminium hydroxide (endotoxin-free) to become CD83/CD1a positive DCs, however, these cells appear to have specifically up-regulated MHC class II and CD86 and this process did not appear to be IL-4 dependent [39]. In both of these situations, PBMC required a 48 h incubation with aluminium hydroxide in order to reveal activation, suggesting that a simple ligand receptor interaction may not explain these observations. As necrotic cell debris has been shown to act as an adjuvant in vitro and in vivo [40], a more likely proposition would be that aluminium hydroxide accumulation could be directly cytotoxic to DCs/macrophages [39], and this may cause bystander activation of DCs.

A number of in vivo studies have also discounted the ability of aluminium adjuvants to directly activate DCs in a TLR dependant fashion. Studies by Schnare et al. [41] demonstrated that the aluminium adjuvants enhance T cell responses independently of the adapter protein MyD88, which plays a central role in TLR signalling and DC activation. Furthermore, the Th1 response induced by FCA was lost in mice deficient for MyD88, leading the authors to conclude that MyD88 signalling, and presumably TLR induced responses are not required to generate Th2 immune responses [41]. In contrast, further studies from the same group demonstrate that aluminium adjuvants fail to have any adjuvant activity when used with antigens that are entirely free of contaminating TLR ligands. When endotoxin-free antigen (human serum albumin) was administered with aluminium adjuvants, no T cell activation ensued, whereas the same antigen/adjuvant preparation when spiked with LPS induced TLR4 dependant T cell responses. The authors conclude that previous studies, for example, those using ovalbumin, are compromised due to the contamination of this antigen with LPS. However, other studies using endotoxin-free antigens formulated with aluminium adjuvants, clearly demonstrate a number of immunological effects, such as T cell expansion and effector function independently of contamination [42].

In contrast to administration of TLR ligands, there is generally a failure to detect increased expression of activation markers on DCs or increased numbers of DCs migrating to draining lymph nodes following in vivo introduction of aluminium adjuvants [43]. Nevertheless, in these circumstances, aluminium adjuvants continues to have adjuvant activity and produce enhanced antigen specific T cell clonal expansion and effector function [42]. Following intraperitoneal injection, aluminium adjuvants do appear to induce priming of splenic B cells, entering a state where they become capable of mobilising Ca^{2+} and activating, rather than undergoing apoptosis, on exposure to antigen. This has been shown to be due to aluminium adjuvant-induced generation of IL-4 by an undefined myeloid population ($Gr1^{+}$, $CD11c^{-/low}$, Class II^{-}, $CD80^{-}$, $CD86^{-}$, $F4/80^{+/-}$, $CD68^{+/-}$) [43]. The significance of this mechanism in generation of T cell responses is unclear, especially given that B cell priming occurs about 6 days following challenge, a time when primary T cell expansion is waning [42]. Presumably, T cells will require activation in the primary lymph organs to alter their chemokine receptor expression and undergo follicular migration prior to interaction with B cells. Therefore, it seems more likely that rather than act as a prime mover in initiating an immune response, B cell presentation later in the response induced by aluminium adjuvants may act to ensure maximal clonal expansion [44].

5. Indirect activation of APCs by aluminium adjuvants

Given the massive heterogeneity of agents with adjuvant activity, it seems more likely that adjuvants may stimulate the production of endogenously synthesised factors, which could then stimulate the activation of DCs. One of the common activities of adjuvants is the ability, in varying degrees, to stimulate local inflammation at the injection site. Indeed, it has been proposed in the past that the induction of local reactions following vaccination is essential for the induction of an effective immune response [9]. As mentioned above, it is the induction of local tissue necrosis observed following the administration of aluminium adjuvants that might consequently induce DC activation [40]. Furthermore, the inflammatory process induced by adjuvants may induce other endogenous agents, for example, TNF, that can cause bystander DC activation. Significantly, one prediction from this model would be an inability to separate the degree of tissue damage induced by an adjuvant from the magnitude of the immune response it can produce. Such a relationship would clearly have significant implications for the development of safe and effective vaccine adjuvants [6]. However, a number of previous studies have attempted, with success, in separating adjuvant activity from toxicity, indicating that this relationship may be more complex than predicted from the danger model [45–47].

Recently, interest has focussed on a histological lesion that may follow immunisation with aluminium containing vaccines and appears to contain persisting aluminium deposits within macrophages in the muscle tissue [48]. This tissue response, referred to as ‘macrophagic myofasciitis’ has been linked to an indeterminate group of symptoms including muscular pain and fatigue, in association with other conditions, including those of an autoimmune nature [48]. Analysis of the route and rate of clearance of radiolabelled (^{26}Al) aluminium adjuvants has demonstrated that while an initial rapid release probably eliminates most of the vaccine-associated ^{26}Al, a longer-term process also occurred that appeared to involve storage in bone [49]. Importantly, these studies failed to evaluate clearance of aluminium from the tissue or draining lymph node [49]. A recent experimental study following intramuscular injection of aluminium phosphate or aluminium hydroxide adjuvanted tetanus/diphtheria vaccines in monkeys has demonstrated persistence of aluminium phosphate at the injection site for up to 3 months and at least 6 months with aluminium hydroxide [50]. The presence of

macrophages at the injection site was also observed, however, these changes were all localised to the injection site and no dissemination was observed, or were behavioural changes or alterations in muscle strength. The authors concluded that while macrophagic myofasciitis may be a normal part of the response to aluminium adjuvanted vaccines, this was not associated with a broader disease [50].

Clearly, the ability of aluminium adjuvants to drive tissue inflammation is not in doubt, although how or if this relates to their adjuvant activity is not clear. Endogenous mediators such as interferons, IL-12, IL-15, TNF and IL-1 have been proposed to activate DC and show adjuvant activity themselves [51–53] or to take part in the activity of certain adjuvants [52,54]. Recently, Sporri and Reis e Sousa investigated the effect of endogenous inflammatory mediators elaborated by TLR activated DCs on bystander DC function. DCs activated in this fashion could up-regulate class II and costimulatory molecule expression, however they were unable to produce IL-12 [55]. These DCs, could induce clonal expansion of antigen specific CD4+ T cells to a similar extent as conventionally (LPS) activated DCs, however, the resulting T cells did not develop into Th1 cells, presumably due to the lack of IL-12 generation. While this would be consistent with the phenotype of an aluminium adjuvant-induced response, significantly, T cells primed in this fashion also failed to help B cell antibody production. The authors suggest that this type of bystander response may be important during inflammatory episodes to reinforce self-tolerance and avoid autoimmunity [56]. Nevertheless, the presence of other host derived factors associated with inflammation and cell death, such as heat-shock proteins [57], uric acid [58] or bradykinin [59] that may not be present in the experimental system described should not be overlooked.

6. Conclusions

Research into vaccine adjuvants has largely been an empirical exercise; nevertheless, vaccination has been the most successful medical intervention in history. Efforts to bring immunological expertise to bear this area have been frustrated by our lack of understanding of the mechanisms that control induction of immune responses. Currently, our efforts in these areas have largely focussed on microbial challenge, an appropriate choice given that our immune systems have evolved to recognise and respond to a variety of unwelcome colonists. Despite these advances, when vaccine developers require an adjuvant for use in clinical medicine, aluminium compounds remain the only choice. Furthermore, existing vaccines that use these adjuvants are likely to remain in use for the long-term foreseeable future. After over 75 years of use, it is appropriate and timely that the mechanism of adjuvant activity of aluminium adjuvants should be elucidated. This knowledge would not only be of basic scientific, but also medical importance, as understanding how aluminium adjuvants potentiates an immune response would facilitate the informed and rational design of new adjuvants as well as the modification of existing adjuvants.

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