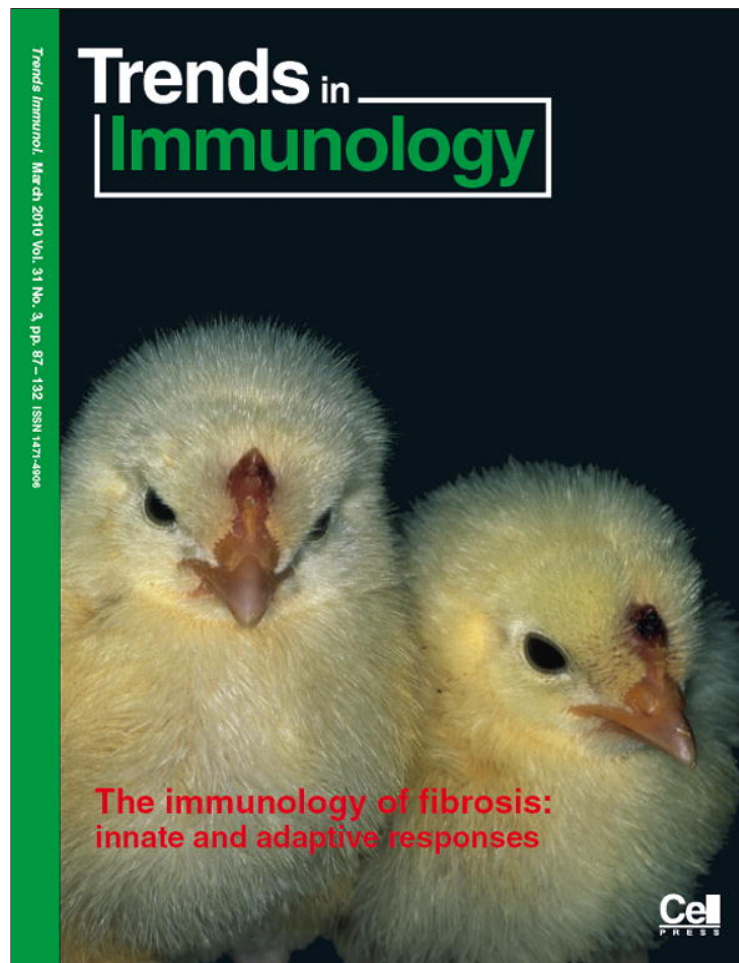


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The immunobiology of aluminium adjuvants: how do they really work?

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Aluminium adjuvants potentiate the immune response, thereby ensuring the potency and efficacy of typically sparingly available antigen. Their concomitant critical importance in mass vaccination programmes may have prompted recent intense interest in understanding how they work and their safety. Progress in these areas is stymied, however, by a lack of accessible knowledge pertaining to the bioinorganic chemistry of aluminium adjuvants, and, consequently, the inappropriate application and interpretation of experimental models of their mode of action. The objective herein is, therefore, to identify the many ways that aluminium chemistry contributes to the wide and versatile armoury of its adjuvants, such that future research might be guided towards a fuller understanding of their role in human vaccinations.

Background

A recent spate of exciting and insightful research papers have, at long last, purported to explain the *modus operandi* of aluminium adjuvants (Al_{ADJ}) [1–7]. Unfortunately, the flurry of review papers that followed the new research have not reached consensus upon the aetiology of the biological activities of Al_{ADJ} [8–10]. Indeed close scrutiny of the new research suggests that an all too liberal application of Occam's razor by scientists and journalists alike was pervasive in them reaching their conclusion that the immunologists' 'dirty little secret' [11] had been revealed. Actually, the recent research, rather than explaining how Al_{ADJ} work, has opened the lid on a Pandora's Box of potential and putative actions of aluminium salts in the context of their use as adjuvants. It has identified many biochemical pathways as potential targets for reactions involving aluminium, and sought to implicate such in immune responses to vaccines that include Al_{ADJ}. The confusion of new information has arisen partly from a recognition of the biological reactivity of aluminium, and partly from the diversity of experimental systems and Al_{ADJ} preparations that were used in past and recent research. There has been a tendency to treat all aluminium salts or preparations as being 'biochemically equivalent' with respect to how physiology reacts to their presence. To date, the majority of attempts to elucidate the mechanism of action of Al_{ADJ} has come from the perspective of immu-

nologists and, possibly, have lacked an understanding of the biological availability of aluminium. Consideration of the bioinorganic chemistry of Al_{ADJ} in light of their immunology should help to consolidate the new information on their modes of action and bring much needed clarity to how they work as clinically approved adjuvants in human vaccinations.

The vaccine and the injection site

The constitution of a vaccine that consists primarily of antigen and Al_{ADJ} is substantially different than that of the physiological milieu into which it is diluted at the injection site. The vaccine preparation is primarily micrometer-sized clusters of nano-sized primary particles of the aluminium salt with which the antigen is associated by adsorption and entrapment [12]. The avidity with which the adjuvant associates with the antigen will depend upon multiple factors, including the form of aluminium salt (usually oxyhydroxide or hydroxyphosphate), the physico-chemical properties of the antigen (including its overall charge and molecular weight), the mode of preparation of the antigen-adjuvant complex (for example, ratio of adjuvant to antigen), and the final solution pH. The latter will usually be around neutral (pH 7.0 ± 0.5), and, along with the highly super-saturated state of the aluminium salt, this will ensure that the concentration of soluble aluminium in the vaccine preparation remains below *ca* 2 μM [12,13]. Similarly, there will be a variable proportion of antigen, often <1% of the total antigen load, that is not associated directly with the adjuvant [14–16], and some of this 'free' antigen may also be in a complex with aluminium. Injection of this vaccine 'soup' usually involves the dilution of *ca* 0.5 mg of total aluminium into the interstitial fluid at the injection site. The interstitial fluid of the receiving tissue is likely to be pH 7.4, to have an ionic composition similar to plasma, and to be rich in nutrients and metabolites related to tissue growth and function. In short, its composition is very different than that of a vaccine preparation, and in the immediate vicinity of the injection site it will be significantly influenced by its mixing with the vaccine. There will also be ingress of plasma and infiltration of blood cells from the disruption of capillaries as the direct result of the physical consequences of an injection. While there will be an immediate limited migration of some of the smaller or non-particulate forms of the vaccine preparation away from the injection site the

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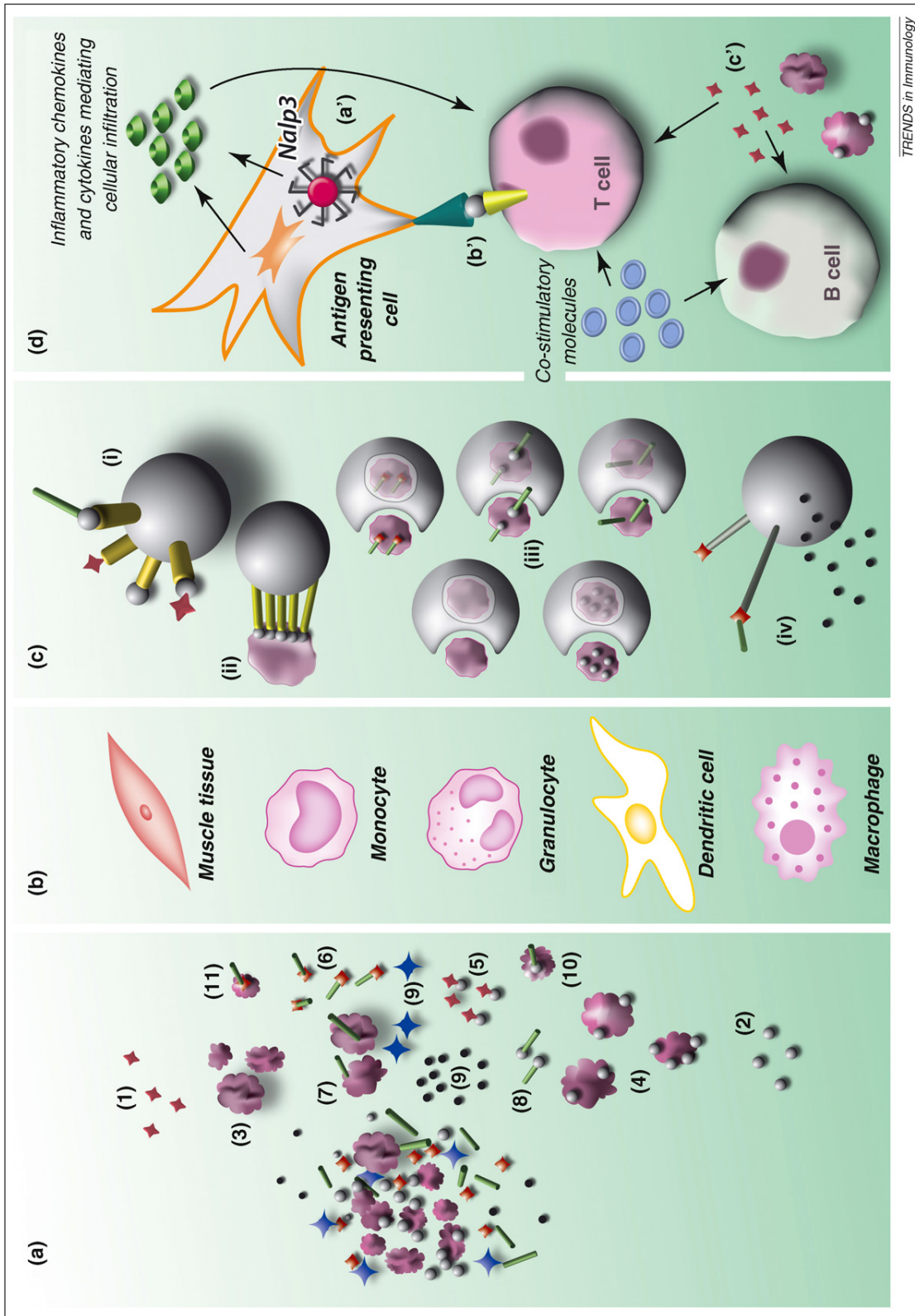


Figure 1. The aluminum adjuvant armory and innate and adaptive immunity. (a) Dilution of the vaccine preparation into the muscle interstitial fluid (MIF) results in an array of potential agonists of the immune cascade, including: (1) Al^{3+} , (2) free antigen (AG); (3) particulate adjuvant (ADJ); (4) ADJ with associated AG; (5) AG-AI complex; (6) MIF ligand-AI complex; (7) ADJ with associated MIF ligand-AG complex; (8) MIF ligand-AG complex; (9) particulate iron (as contaminant of adjuvant) either free or with adsorbed AI/AG and resultant reactive oxygen species (ROS); (10) ADJ with associated MIF ligand-AI complex; (11) ADJ with associated MIF ligand-AG complex. MIF ligands might include biomolecules such as; ATP, albumin, transferrin, citrate, fibrinogen. (b) The array of agonists act upon a number of cell types including, the resident muscle tissue (potentially causing necrotic and/or apoptotic cell death) and infiltrating innate cells such as, monocytes (potential for Al_{ADJ} -induced eosinophilia acting directly on B cells), macrophages (are known to persist for long periods close to the injection site and may be characterised by inclusions of Al_{ADJ}) and dendritic cells (DC). The latter may be the major antigen presenting cell (APC). (c) There are myriad possible modes of interaction between agonists and innate cells including: (i) toll-like receptor (TLR) binding of AG², AG-AI complex⁵, MIF ligand-AG complex⁵, Al^{3+} ; (ii) multiple TLR binding of AG-ADJ⁴, AG-ADJ⁴, MIF ligand-ADJ⁷, MIF ligand-AI complex-ADJ¹¹, MIF ligand-AG complex-ADJ¹⁰; (iv) direct⁶ or indirect⁸ binding of Al^{3+} by membrane receptors and extracellular (lipid membrane) or intracellular (nucleus) activity of ROS⁹. (d) APCs activate adaptive immunity through; (a) Nalp3 inflammasome dependent or independent release of chemokines and cytokines (green saucers) including IL-1 β and IL-18; (b) AG presentation by MHC to T cell receptor combined with co-stimulatory molecules; (c) direct action of ADJ and/or Al^{3+} on B/T cells. The superscripts refer to the numbers in parentheses in the figure.

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majority of adjuvant and antigen will, during the following hours, remain close to the site where the 'mix' of vaccine, interstitial fluid and plasma will initiate a local reaction.

The soluble forms of aluminium that are delivered to the injection site in the vaccine, primarily aluminate ($\text{Al}(\text{OH})_4^-$) in equilibrium with Al^{3+} and its hydrolysis products [13], will be the first to migrate away from the injection site as they dilute into the continuously replenishing interstitial fluid. Aluminium salts are slightly more soluble at pH 7.4 than pH 7.0 and this solubility gradient will drive, if slowly, the continued dissolution of particulate aluminium. A burgeoning concentration of Al^{3+} will be available for binding by soluble ligands within the interstitial fluid (e.g. amino and carboxylic acids and proteins such as albumin and fibrinogen) and anchored ligands (e.g. phosphate and carboxylate groups) within cell membranes and other structures. These interactions will, in addition to the pH gradient, accelerate the dissolution of particulate aluminium, although the rate will remain comparatively slow, because of the kinetic inertia of the aluminium salts, and protection of dissolution sites on the aluminium salt by adsorbed and occluded antigen. Thus, while a small proportion of injected aluminium will be present close to the injection site in a rapidly biologically available form, Al^{3+} , the majority of injected aluminium will be present as particulates both with and without associated antigen.

Aluminium as ammunition

The adjuvant activity of aluminium salts could potentially be ascribed to either soluble or insoluble (particulate) aluminium, or as a combined response to both forms of aluminium. The biologically reactive form of aluminium is primarily Al^{3+} and this small and highly electropositive hydrated ion is avidly bound by oxygen and fluoride-based functional groups [17]. The latter are probably of lesser importance in aluminium biochemistry, although aluminium fluoride complexes are potent agonists of G proteins [18]. Many oxygen-based functional groups and ligands bind Al^{3+} in preference to their usual metal-cofactors; for example ATP will always bind Al^{3+} in competition with Mg^{2+} [19]. Reactions between biomolecules and adjuvant-derived Al^{3+} will be rapid and involve aluminium being transported away from the injection site. A seminal study using ^{26}Al -labelled aluminium oxyhydroxide and aluminium hydroxyphosphate adjuvants demonstrated the presence of ^{26}Al in blood within 1 h of their intramuscular injection in rabbits [20]. This research highlighted the lability of aluminium when administered as an adjuvant, and implicated the activity of Al^{3+} either close to or distant from the injection site in their mode of action. However, the prevalent and most persistent form of aluminium at the injection site will be (slowly dissociating and dissolving) particulates of the order of 1-20 μm in size. Their role in adjuvant activity would include them being (i) a consistent source of Al^{3+} , (ii) a steady supply of desorbed antigen, (iii) a surface that presents a dense population of antigen to some forms of recognition molecules, and (iv) a collection of optimally-sized particles for phagocytosis by resident and infiltrating cell types. While Al^{3+} is the biologically reactive form of aluminium, being available for complexation by multifar-

ious biomolecules, an Al_{ADJ} 's armoury also includes ammunition in the form of particulates that thereby widen the scope of potential biological targets (see Figure 1).

Biological targets of aluminium adjuvants

While the total concentration of aluminium at the injection site will be high (mM), the availability of cytotoxic Al^{3+} (nM - μM) is unlikely to be high enough, even over a prolonged exposure, to induce necrotic cell death [17]. Similarly, the particulate forms of aluminium found in clinically approved adjuvants are not expected to exert a 'physical' or 'morphology-based' toxicity on, for example, cell or lysosomal membranes [21] as has been suggested for silica [7] or crystals of monosodium urate [22]. Though often inferred, there is very little direct evidence in the scientific literature [23] of the acute toxicity of clinically approved Al_{ADJ} in tissues in the vicinity of the injection site [1]. With this in mind, it is important to consider that infiltrating phagocytes will find an unlimited diet of particulate Al_{ADJ} at the injection site and will 'eat' until they die, thereby releasing various damage-associated molecular patterns (DAMPs). The next line of phagocytosing cells will thus encounter an environment rich in both particulate Al_{ADJ} and DAMPs; this would increase the possibility of activation of the Nalp3 inflammasome, and the production of IL-1 β , and thus, induction of inflammation and increased recruitment, activation, and maturation of immune competent cells (see Box 1).

The cellular response to exposure to Al^{3+} is known to be biphasic, i.e. stimulatory at low, and inhibitory at high, concentrations. At the concentrations expected in the interstitial fluid proximal to the injection site, Al^{3+} would exert stimulatory, as opposed to inhibitory, effects in resident cells and tissues [17]. The nature of such effects might be gleaned from examples of exposure of other cell types to aluminium. For example, exposure of human brain cells in primary culture to nM concentrations of Al(III) induced significant upregulation of expression of genes that promote inflammatory signalling (e.g. *IL-1 β* precursor) and apoptosis (e.g. *DAXX*) [24]. Similar pro-inflammatory effects (e.g. upregulation of NF- κB) following chronic exposure to Al(III) have been observed in human glioblastoma [25], and in a wide range of animal models [26-30]. There is strong evidence that many of the pro-inflammatory effects of chronic systemic exposure to aluminium are

Box 1. Inflammatory mediators

The inflammation process mediates a link between the innate and adaptive immune response by providing an environment essential for the induction of an adaptive immune response. Mediators connecting the innate and adaptive immune response are components facilitating cell infiltration and differentiation/activation signals. Pro-inflammatory mediators can be exemplified by cytokines, chemotactic cytokines/chemokines, reactive oxygen species (ROS) and nitric oxide (NO).

Major pro-inflammatory cytokines:

IL1-alpha, IL1-beta, IL6, and TNF-alpha.

Chemoattractants:

IL8, MCP 1, -4, MIP-1, RANTES, GRO- α , - β , - γ

Reactive Oxygen Species (ROS) and NO:

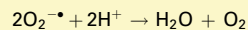
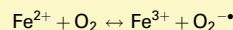
Superoxide anion, hydroxyl radical, hydrogen peroxide, peroxynitrite and nitric oxide

mediated via the formation of reactive oxygen species (ROS) [24,31]. Since aluminium is a powerful pro-oxidant, possibly through its binding by the superoxide radical anion [32], it would be expected to promote oxidative events at the injection site. It is of note that clinically approved Al_{ADJ} are contaminated with significant concentrations (ppm) of iron. Since aluminium, under physiological conditions, can both reduce Fe(III) to Fe(II) and promote the auto-oxidation of the latter [33], then the combination of iron and aluminium will potentiate the formation and activities of ROS at and close to the injection site (see Box 2). The known role of aluminium, as adjuvant and otherwise, in mediating the formation and release of IL-1 β and IL-18 may also involve extracellular signalling via ATP. Extracellular ATP is implicated in the release of pro-inflammatory cytokines through its action at one or more types of P2 receptor in a number of immune-responsive cell types, including macrophages [34,35]. Aluminium has been shown to potentiate the activity of extracellular ATP either by prolonging the lifetime of the nucleotide-receptor complex or by reducing the rate of its hydrolysis by ectonucleotidases [36,37]. A possible effect of aluminium on the rate of hydrolysis of ATP would be to boost the immune response through blocking T regulatory cell function [38,39]. In further support of a role for Al-ATP in modulating the immune response, it is well known that aluminium toxicity in plant roots is often manifested as enhanced efflux of potassium [40], the latter being a further signal for activation of caspase-1 and release of IL-1 β and IL-18 [41]. In fact experiments which demonstrated the promotion of B cell immune responses by the action of Al_{ADJ} in the absence of adsorbed antigen showed aluminium-induced increases in intracellular calcium [42] which is an effect which tallies with the known aluminium-induced increased expression of the gene for cytosolic phospholipase A₂ (cPLA₂) [24]. Clearly, there are myriad possibilities for Al³⁺_(aq) to influence innate (and adaptive) immunity, possibly [2,4], but not exclusively [5], involving the activation of the Nalp3 inflammasome, and, importantly, without the prerequisite of cell death [1].

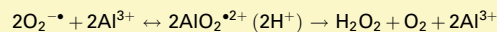
Distinct roles for particulate aluminium in potentiating the immune response to antigen are less easily defined. Phagocytosis of Al_{ADJ} by innate cells is not by itself sufficient to result in the subsequent intracellular lysis of the phagolysosome and activation of the Nalp3 inflammasome. As already mentioned, particulates of Al_{ADJ}, including the poorly crystalline boehmite of commercial aluminium hydroxide preparations, cannot disrupt membrane structures through 'morphology-based' toxicity [21]. However, it is possible that the integrity of phagolysosomes might be disrupted if their contents were acidified by an active process that thereby promoted the dissolution of the particulate aluminium and release of membrane-damaging Al³⁺_(aq) [43]. The release of aluminium into the cell cytosol could then result in activation of the Nalp3 inflammasome through, for example, a pro-oxidant mechanism [44]. Similar mechanisms whereby both soluble and particulate metals activated the Nalp3 inflammasome have been demonstrated in macrophages [45]. Particulate aluminium, following its phagocytosis by an antigen-presenting cell, might potentiate an immune response

Box 2. Aluminium facilitates iron-driven biological oxidation

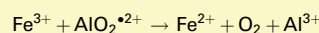
The mechanism that is proposed to underlie this effect involves the formation of the aluminium superoxide semi-reduced radical ion, AlO₂^{•2+}, that acts as a pro-oxidant in both catalyzing the formation of hydrogen peroxide, H₂O₂, and reducing Fe³⁺ to Fe²⁺.



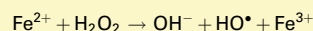
and



and



thereby facilitates the reaction



Redox cycles are integral components of adjuvant-mediated pro-inflammatory signalling [62], and are clear targets for potentiation by aluminium.

through its delivery of a significant population of associated antigen to T cells in lymph nodes [46,47]. The concomitant release of the adjuvant aluminium as Al³⁺_(aq) might then act in a co-stimulatory manner, perhaps as has been previously noted for B cells [42]. The classical depot mechanism of action of Al_{ADJ} is usually described as the slow but consistent release of antigen, whereafter the antigen is processed and presented by innate cells to T cells as an MHC-antigen complex. What is less clear is if pattern recognition receptors (PRR) on innate cells can 'sense' and bind antigen that is still associated with the adjuvant [48]. If this were possible, then the recruitment of PRRs at high density in the cell membrane of innate cells could result in a more 'efficient' immune response both with respect to subsequent cytokine secretion, and the processing and presentation of MHC-antigen (see Figure 1).

There are other biological responses to Al_{ADJ} that could impact upon their action as adjuvants. Aluminium compounds are used in a vast number different industrial applications including as surface modifiers and as catalysts [49]. All particulate forms of aluminium have the potential to act as surfaces for adsorption or entrapment of biomolecules, just as they do in vaccine preparations, and such surfaces could further act as templates for biomolecular ordering and catalysis of biochemical reactions. They could act in this way *in situ* at the injection site, following desorption of associated antigen, or away from the injection site, for example in lymph nodes, either having been transported as particulates by phagocytosis or conceivably having been re-precipitated as amorphous aluminium hydroxide [50]. Thus it is quite possible that the adjuvant activity of an aluminium salt may not be restricted to its enhancement of the antigenicity of the co-administered antigen, but might also act to enhance the antigenicity of biomolecules that are already present in the interstitial fluid of the recipient of the vaccine [51]. Such 'naturally-occurring' foreign biomolecules may not, in the absence of the residual Al_{ADJ} material, be present in sufficient numbers to trigger an immune response. Alternatively, they

may not expose antigenic determinants in their native 3-dimensional structure that could be exposed following their adsorption to Al_{ADJ}. In relation to this possible 'indirect adjuvanticity' there are burgeoning examples in the scientific literature of aluminium salts inducing sensitization to substances that might not normally be considered as antigens. For example, such effects may contribute towards allergies to foods [52].

Another biological response to Al_{ADJ} that has apparently gone unnoticed is the recognition that aluminium itself might also be antigenic. Monoclonal antibodies that were raised against an aluminium-BSA (bovine serum albumin) immunogen were shown to recognise both protein and non-protein bound aluminium under physiological solutions [53]. These antibodies were then used successfully to identify aluminium in human brain tissue [54]. The propensity for metals to act as antigens when conjugated to proteins, does not appear to be unique to aluminium [55], and this property should be recognized as a potential contributor to the adjuvant activity of aluminium salts. In the modern world that has been coined 'the aluminium age' [56], all humans are exposed to aluminium throughout their lives from conception, through birth and to death. Aluminium accumulates throughout the body with age [57], and each time an individual receives a vaccination that includes an Al_{ADJ}, there is the potential to raise an immune response against both the adjuvant and any significant body stores of aluminium. There are a burgeoning number of reports of adverse reactions to vaccinations that include Al_{ADJ}, and some of these atypical events might be explained by the apparent antigenicity of aluminium itself [58].

When an aluminium adjuvant is not an aluminium adjuvant

In spite of the significant efforts of Stanley Hem and colleagues [12,59], researchers have continued to treat all aluminium salts as 'biochemical equals' with respect to their modes of action as adjuvants. Few apart from Hem have appreciated that the detailed mechanism of action of the two clinically approved Al_{ADJ}, commonly referred to as aluminium hydroxide and aluminium phosphate, will be different from each other, while that of the non-clinically approved experimental adjuvant material known as Imject[®] Alum will be radically different from either of the products approved for use in humans. Problems have arisen where experiments *in vitro* and in animals have used Imject[®] Alum as a model of clinically approved adjuvants in human vaccinations. It is a problem in that while this product is an effective adjuvant, its formulation and physico-chemistry is that of an antacid. It is composed of equal weights (40 g/L) of aluminium hydroxycarbonate and magnesium hydroxide. The latter is not included in clinically approved Al_{ADJ} and is included in Imject[®] Alum, according to a personal e-mail correspondence with the manufacturers, "to improve the immune response to the adjuvant". The suggested improved response could be due to magnesium's myriad functions in physiology many of which relate to functioning of the immune system [60] (see Box 3). In addition, magnesium is a known cardioprotectant, and this might explain the apparent atheroprotective

Box 3. Magnesium and the immune system

Magnesium, as Mg²⁺, is the second most abundant cation in cellular systems, and has myriad roles in biochemical systems [63]. Magnesium salts have been used as adjuvants in the treatment of heart disease [64] and asthma [65], and in anaesthesia [66]. While magnesium has been implicated in various roles in the immune system [60], the mechanisms underlying such remain to be fully elucidated. For example, magnesium is involved in cellular proliferation [67], and this may have implications for cellular differentiation in innate immunity. Magnesium is generally protective against pro-inflammatory conditions [68,69] and may exert its anti-inflammatory effects through reducing the secretion of cytokines [70] or regulation of the activity of NF-κB [71]. Magnesium is also the natural biochemical antagonist to the pro-inflammatory aluminium [72], and this, alone, may underlie many of its adjuvant-related biochemical effects.

effects of Imject[®] Alum [51]. Another way by which the presence of a high concentration of magnesium might improve the adjuvant effect of Imject[®] Alum is through an amelioration of the effects of administering an aluminium salt that is significantly more soluble, and less kinetically inert, than either aluminium oxyhydroxide or aluminium hydroxyphosphate. To be an effective antacid, aluminium hydroxycarbonate must respond to changes in [H⁺] by the rapid release of soluble forms of aluminium (Al³⁺_(aq) ↔ Al(OH)₄⁻_(aq)) to buffer any pH change. The dilution of Imject[®] Alum into interstitial fluid will promote any biological response that is primarily mediated via Al³⁺_(aq). Higher concentrations of biochemically reactive aluminium could result in inhibitory, as opposed to stimulatory, effects, for example, through inhibition of the activity of NADH oxidase [61]. Such effects would not necessarily manifest as an immunopotential. However, the co-presence of a significant molar excess of biologically reactive Mg²⁺ would be expected to provide partial protection against the inhibitory activities of Al³⁺_(aq) [13,19,56], and thereby mediate at least some of the improved adjuvanticity that the manufacturer of Imject[®] Alum has attributed to magnesium hydroxide (see Box 3). The higher solubility of aluminium hydroxycarbonate in Imject[®] Alum could also result in significant precipitation of amorphous aluminium hydroxide and its subsequent endocytosis by cells either close to or away from the injection site. This particulate aluminium is chemically distinct from the parent material of the adjuvant and could, following endosomal acidification and lysis, be released into cell cytosol where it would have the potential to be cytotoxic. Thus, for equivalent concentrations of total aluminium in applied adjuvants, necrotic cell death would be more likely for Imject[®] Alum than for aluminium oxyhydroxide or aluminium hydroxyphosphate. Therefore, for excellent reasons already pointed out by Hem [12,59], and emphasised further herein, Imject[®] Alum should not be used as a model for understanding the *modus operandi* of clinically approved Al_{ADJ}.

Conclusions

Recent detailed and insightful research into the possible mechanisms of action of Al_{ADJ} has progressed significantly our understanding of the biochemistry of aluminium. While the pro-inflammatory effects of chronic aluminium

intoxication have been known for many years, there was little understanding of the underlying aetiology. There are now strong precedents for the involvement of the Nalp3 inflammasome in the known toxicity of aluminium as well as other Nalp3 inflammasome-independent effects which are mediated through antigen presenting cells and directly or indirectly upon B and T cells. However, does the new research explain the mechanism of action of clinically approved Al_{ADJ}? The answer is 'probably not,' as the appropriate experiments, particularly in humans, remain to be carried out. Taking all evidence as a whole, someone with a 'feeling' for the biological chemistry of aluminium might still favour one or a combination of mechanisms relating to the classical 'depot' effect as the likely primary mode of action of 'true' Al_{ADJ}, with the one proviso that we, as individuals, will not all respond in an identical manner, either in the short or long term, to injection of aluminium into our tissue.

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