



Review article

Critical analysis of reference studies on the toxicokinetics of aluminum-based adjuvants[☆]

Jean-Daniel Masson^{a,1}, Guillemette Crépeaux^{a,b,1}, François-Jérôme Authier^a, Christopher Exley^c, Romain K. Gherardi^{a,*}

^a INSERM U955 E10, Biologie du système neuromusculaire, Faculté de Médecine, Université Paris Est Créteil, Créteil 94010, France

^b Génétique médicale comparée des affections neuromusculaires, Ecole Nationale Vétérinaire d'Alfort, 7 Avenue du général de Gaulle, 9400 Maisons-Alfort, France

^c The Birchall Centre, Lennard-Jones Laboratories, Keele University, Staffordshire ST5 5BG, UK

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ABSTRACT

We reviewed the three toxicokinetic reference studies commonly used to suggest that aluminum (Al)-based adjuvants are innocuous. A single experimental study was carried out using isotopic ²⁶Al (Flarend et al., Vaccine, 1997). This study used aluminum salts resembling those used in vaccines but ignored adjuvant uptake by cells that was not fully documented at the time. It was conducted over a short period of time (28 days) and used only two rabbits per adjuvant. At the endpoint, Al elimination in the urine accounted for 6% for Al hydroxide and 22% for Al phosphate, both results being incompatible with rapid elimination of vaccine-derived Al in urine. Two theoretical studies have evaluated the potential risk of vaccine Al in infants, by reference to an oral “minimal risk level” (MRL) extrapolated from animal studies. Keith et al. (Vaccine, 2002) used a high MRL (2 mg/kg/d), an erroneous model of 100% immediate absorption of vaccine Al, and did not consider renal and blood-brain barrier immaturity. Mitkus et al. (Vaccine, 2011) only considered solubilized Al, with erroneous calculations of absorption duration. Systemic Al particle diffusion and neuro-inflammatory potential were omitted. The MRL they used was both inappropriate (oral Al vs. injected adjuvant) and still too high (1 mg/kg/d) regarding recent animal studies. Both paucity and serious weaknesses of reference studies strongly suggest that novel experimental studies of Al adjuvants toxicokinetics should be performed on the long-term, including both neonatal and adult exposures, to ensure their safety and restore population confidence in Al-containing vaccines.

1. Introduction

Vaccination helped with the eradication of smallpox, a 99% decline in poliomyelitis between 1988 and 2003, and a 40% decrease in measles cases between 1999 and 2003 worldwide, as well as a decrease in cases of mumps of 859 to 9 per 100,000 inhabitants between 1986 and 2013 in France [1]. The maintenance of good vaccination coverage, i.e. a high rate of vaccinated persons in the population, is necessary to avoid the resurgence of other infectious diseases, as was observed for pertussis or rubella, with a double benefit, both individually and collectively, by reducing the number of people who can transmit infectious diseases [1].

Although the success of many vaccines has been amply demonstrated, a growing public distrust of vaccination has emerged in recent years. This reluctance, of varying degrees, appears concomitantly with an expanding

global World Health Organization (WHO) policy for burgeoning vaccination programs with > 120 new vaccines currently being developed and an annual growth of 20% of vaccine business is expected, realizing a turnover which has increased from 5 to 43 billion dollars between 2000 and 2016, and will be > 100 billion dollars in 2025 [2].

Unlike conventional medicines, vaccines are administered to healthy subjects that need to be convinced of their value and safety. In this context, the vaccine issue has become a major societal issue, leading to the establishment of a national citizen consultation on vaccination chaired by Alain Fischer in France [3]. According to the findings of its final report of 30th of November 2016, several factors contribute to mistrust of vaccination, *especially*:

- Suspicions of collusion between health authorities and the drug industry as a result of mediated scandals;

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* Corresponding author at: Centre Expert de Pathologie Neuromusculaire, Hôpital Henri Mondor, 94010 Créteil, France.

E-mail address: romain.gherardi@aphp.fr (R.K. Gherardi).

¹ These authors contribute equally to this work.

- The disappearance of many infectious diseases that question the appropriateness of continuing vaccination;
- The issue of adjuvants in vaccines;
- The position of doctors who complain of a lack of training to convince reluctant patients;
- The complexity of the vaccination course (mandatory medical prescription, pharmacy purchase of the vaccine, medical vaccination, etc.);
- Lack of information from doctors on the immunization status of their patients (health book lost or not presented);
- Health crises (mediator, contaminated blood, etc.) and the insufficient responsiveness of the answer and the commitment of the public authorities which have left the field open to anti-vaccination propaganda [3].

A key question in the debate on vaccine safety concerns the adjuvants, compounds essential for strong and lasting immunization [4]. The controversy focuses on the aluminum salts which were empirically introduced by Alexander Glenny as adjuvants to vaccines in 1926 [5]. It has resulted in various actions brought by patient associations [6,7], publication of books for the general public, either critical [8] or reassuring [9], scientific blogs [10], drafting of institutional technical reports [4,11–13], and holding of parliamentary initiative discussion meetings [14,15]. Although the principle of vaccination has never been questioned during these exchanges, the exact degree of safety of aluminum-containing vaccines has remained the subject of persistent disagreement.

The occurrence of myalgia and arthralgia, chronic fatigue and neurological disorders following multiple injections of aluminum-containing vaccines against hepatitis B, tetanus and human papilloma virus (HPV) has been reported in many countries: Australia [16], Canada [17,18], Denmark [19,20], France [21–23], United Kingdom [24,25], Italy [26], Israel [27], Japan [28–29], Mexico [30], Portugal [31], and USA [32]. Nevertheless, beyond the temporal association, the existence of a causal link remains debated. For vaccination against HPV for example, the risk of occurrence of adverse events, which may form part of one or more of the clinical entities [19] - chronic fatigue syndrome (CFS), regional pain syndrome (RPS), orthostatic postural tachycardia syndrome (POTS) – emerges from an epidemiologic point of view [33]. A systematic cross-sectional study of 12 published studies showed a slight increase of adverse events in the HPV-vaccinated group, but this information must take account of the quasi-systematic use of control groups that received aluminum adjuvants in the form of a placebo containing the adjuvant or, more rarely, the hepatitis A vaccine (11 of the 12 publications analyzed, comprising 29,533 of the 29,600 patients studied) [34]. Despite this major bias [35], European Medicines Agency (EMA) issued a negative opinion on the existence of an association between HPV-vaccination and increasing of adverse events [36]. Some pharmaco-epidemiological studies were seemingly in support of this opinion [37,38], but having focused on most specific auto-immune diseases, they have excluded CFS, RPS, and POTS from their investigations. The EMA's decision caused strong dissatisfaction of Cochrane Nordic and a complaint was lodged against EMA [39]. The question of the existence of a causal link, and thus of an authentic adjuvant syndrome [40,41], may never be resolved by epidemiological approaches [42]. The performance of epidemiology to establish causality is notoriously limited, as it can be conceived for multi-systemic effects in the more or less long term of low cumulative doses administered in a context of multiple exposures. Failing this, the debate can be enlightened only by establishing the existence or not of an unequivocal biological plausibility of a causal link.

To date, aluminum adjuvants per se have, perhaps surprisingly, not been the subject of any official experimental investigation, and this being in spite of the well-established neurotoxicity of aluminum. The WHO also notes: "Adjuvant safety is an important and neglected field. Since adjuvants have their own pharmacological properties, which

might affect both the immunogenicity and the safety of vaccines, safety assessment is essential" [43]. For its part, the National French Academy of Pharmacy asked that studies on the safety of the aluminum-based adjuvants be carried out taking into account a set of parameters so far little studied, which can contribute to the appearance of risk [13]. In the following review, we have examined in detail in the light of recent findings the few articles of classical toxicokinetics in the literature that serve as a reference for health regulators and industrialists to apparently confirm the safety of aluminum adjuvants.

2. Generality on Al adjuvants

The two main aluminum salts used as adjuvants are Al oxy-hydroxide (AlOOH, Alhydrogel®) and Al hydroxyphosphate (AlOHP₄, Adju-Phos®). They are present in about 60% of human vaccines (Table 1) and veterinary vaccines [44]. The oxy-hydroxide form is the most widely used adjuvant in vaccines distributed in France (the most commonly used vaccines against hepatitis B, hepatitis A, or tetanus, many other vaccines, as well as products for immunotherapy subcutaneous desensitization). For HPV vaccines, the adjuvants are Al-oxy-hydroxide for the divalent 16/18 Cervarix® (combined with a second adjuvant, monophosphoryl lipid A, detoxified derivative of lipopolysaccharide [45]), and amorphous Al hydroxyphosphate sulfate for the quadrivalent 6/11/16/18/ Gardasil® (an adjuvant more immunostimulating than conventional aluminum-based adjuvants) [46].

The two major types of aluminum adjuvant strongly potentiate the production of antibodies (humoral response by activation of CD4 + Th2 lymphocytes and B-cell priming) and not, or very little, production of cytotoxic T lymphocytes. The mechanisms involved are still incompletely understood [47,48]. The Food and Drug Administration (FDA) empirically fixed the authorized level of adjuvant at 0.85 mg of aluminum per dose of vaccine, based on results showing a good adjuvant effect at this concentration (according to Joan May, FDA/CBER, quoted in [49]).

The two Al-adjuvants have different physicochemical properties in the native state. The oxyhydroxide (commonly called Al hydroxide) has a crystalline morphology, known as Boehmite, while hydroxyphosphate (commonly called Al phosphate) is amorphous. Al hydroxide is composed of nanoparticles of about 2.2 nm × 4.5 nm × 10 nm which spontaneously form micron-sized aggregates having a nano-fibrous appearance under transmission electron microscopy [50,51]. This adjuvant is highly hydrated, forming a stable gel whose antigenic adsorption capacities are uniformly high. Hydrostatic interactions and exchange of hydroxyl groups with phosphate are the main forces explaining the adsorption at the surface of the adjuvant. Al phosphate has fewer hydroxyl groups and therefore its antigenic adsorption capacities are lower than those of Al hydroxide. Al hydroxide has a positive surface charge, Al phosphate a negative charge. The kinetics of biodisposition of the two adjuvants are also significantly different: Al hydroxide is much slower solubilized, more avidly internalized and less toxic to the phagocytic cells [51] than Al phosphate, suggesting notable differences in the reactions of the two adjuvants during the interactions with phosphate, organic acids, protein environments and immune cells encountered in vivo.

3. Critical analysis of reference articles on the toxicokinetics of Al adjuvants

3.1. Study of absorption and elimination of vaccine aluminum [52]

For a long time specialized international meetings have held that Al injected by the vaccine route was essentially rapidly eliminated from the body in the urine [53] and this message was relayed by general public official information sites, until recent withdrawal [54]. This claim has its roots in studies from the 1990s using a new technique to study Al toxicokinetics. Indeed, until 1990, it was difficult to know the

Table 1
Aluminum adjuvant-containing vaccines licensed for human use (2013).

Vaccine's name	Laboratory	Aluminum	Other adjuvant
<i>Both bacteria and viruses</i>			
Diphtheria. Tetanus. Acellular pertussis. Poliomyelitis. <i>Haemophilus influenzae</i> B and hepatitis B			
InfanrixHexa	GSK	Al-phosphate: 0.3 mg/dose Al-hydroxide: 0.5 mg/dose (0.5 ml)	
Diphtheria. Tetanus. Acellular pertussis. Poliomyelitis and <i>Haemophilus influenzae</i> B			
InfanrixQuinta	GSK	Al-hydroxide: 0.5 mg/dose (0.5 ml)	
Pentavac	Sanofi Pasteur MSD	Al-hydroxide: 0.3 mg/dose (0.5 ml)	
Diphtheria. Tetanus. Acellular pertussis and poliomyelitis			
DTCaPolio			
InfanrixTetra	GSK	Al-hydroxide: 0.5 mg/dose (0.5 ml)	
Tetravac acellulaire	Sanofi Pasteur MSD	Al-hydroxide: 0.3 mg/dose (0.5 ml)	
dTcaPolio			
Boostrixtetra	GSK	Al-hydroxide: 0.3 mg/dose + Al-phosphate: 0.2 mg/dose (0.5 ml)	
Repevax	Sanofi Pasteur MSD	Al-phosphate: 0.33 mg/dose (5 ml)	
Diphtheria. Tetanus and poliomyelitis			
Revaxis	Sanofi Pasteur MSD	Al-hydroxide: 0.35 mg/dose (0.5 ml)	
<i>Bacteria</i>			
Meningococcus			
Meningococcus C			
Meningitec	Pfizer Holding	Al-phosphate: 0.125 mg/dose (5 ml)	
Menjugatekit	Novartis Vaccines and Diagnostics	Al-hydroxide: 0.3 to 0.4 mg/dose (0.5 ml)	
Neisvac	Baxter	Al-hydroxide: 0.5 mg/dose (0.5 ml)	
Meningococcus B			
Bexsero	Novartis Vaccines and Diagnostics	Al-hydroxide: 0.5 mg/dose (0.5 ml)	
Pneumococcus			
Prevenar 13	Pfizer Holding	Al-phosphate: 0.125 mg/dose (5 ml)	
Pasteur tetanic vaccin	Sanofi Pasteur MSD	Al-hydroxide: 0.6 mg/dose (0.5 ml)	
<i>Viruses</i>			
Hepatitis B			
Engerix 10 µg/0.5 ml	GSK	Al-hydroxide: 0.25 mg/dose (0.5 ml)	
Engerix 20 µg/1 ml	GSK	Al-hydroxide: 0.5 mg/dose (1 ml)	
HBVAXPRO 5 µg/0.5 ml	Sanofi Pasteur MSD	Al-hydroxyphosphate sulfate 0.25 mg/dose (0.5 ml)	
HBVAXPRO 10 µg/1 ml	Sanofi Pasteur MSD	Al-hydroxyphosphate sulfate 0.5 mg/dose (1 ml)	
HBVAXPRO 40 µg/1 ml	Sanofi Pasteur MSD	Al-hydroxyphosphate sulfate 0.5 mg/dose (1 ml)	
GenHevac B Pasteur	Sanofi Pasteur	Al-hydroxide ≤ 1.25 mg/dose (1 ml)	
Hepatitis A			
Avaxim adult	Sanofi Pasteur	Al-hydroxide: 0.3 mg/dose (0.5 ml)	
Havrix 1440 U/1 ml adult	GSK	Al-hydroxide: 0.5 mg/dose (1 ml)	
Havrix 720 U/0.5 ml infant and pregnant women	GSK	Al-hydroxide: 0.25 mg/dose (0.5 ml)	
Tick-borne encephalomyelitis			
Ticovac 0.5 ml adult	Baxter	Al-hydroxide: 0.35 mg/dose (0.5 ml)	
Ticovac 0.25 ml infant	Baxter	Al-hydroxide: 0.17 mg/dose (0.25 ml)	
Encepur	Novartis Vaccines and Diagnostics	Al-hydroxide: 0.3 to 0.4 mg/dose (0.5 ml)	
Japanese encephalitis			
Ixiaro		Al-hydroxide: 0.25 mg/dose (0.5 ml)	
Human papillomavirus			
Cervarix	GSK	Al-hydroxide: 0.5 mg/dose (0.5 ml)	3-O-desacyl-4'-monophosphoryl lipid A (50 µg)
Gardasil	Sanofi Pasteur MSD	Al-hydroxyphosphate sulfate 225 µg/dose (0.5 ml)	
Both hepatitis A and B			
Twinrix adult	GSK	Al-hydroxide: 0.05 mg/dose + Al-phosphate: 0.4 mg/dose (1 ml)	
Twinrix infant	GSK	Al-hydroxide: 0.025 mg/dose + Al-phosphate: 0.2 mg/dose (0.5 ml)	

precise fate of Al in vivo, since it was not possible to differentiate administered Al from Al obtained from other forms of exposure or from external contamination of the samples. The use of ^{26}Al , a low-level radioactive isotope, which is distinct from the natural ^{27}Al , has allowed the detection of very small quantities of Al (10^{-17} g) using accelerator mass spectrometry [55].

Priest et al. [56] were the first to inject intravenously (IV) ^{26}Al citrate, a soluble form of aluminum, into a healthy volunteer to study the

toxicokinetics of aluminum in a human. They observed that more than half of the injected aluminum had left the bloodstream after 15 min and < 1% remained in the bloodstream after two days. On day 13, 83% of the injected dose had been excreted in urine and 1.8% had been excreted in feces [56]. The remaining 15% in the organism after that date then declined very slowly as the retention of ^{26}Al was still 4% after 3 years. Similar results were reported in 6 other healthy volunteers, with significant inter-individual variations in the degree of retention of

aluminum [57]. This work thus showed a multiphase elimination of the circulating Al, comprising an initial rapid elimination phase, followed by phases of elimination which are much slower. Multiple environmental exposures will thus favor the progressive accumulation of aluminum in the body during the life of an individual [56]. It is essential to take into account that in these preliminary toxico-kinetic studies, neither the form of aluminum (soluble) nor the route of administration (IV) corresponded to the vaccine situation, where aluminum is subcutaneously (SC) or intramuscularly (IM) injected in nano/microparticle form. The point is crucial: the dynamics of Al adjuvants have very little relevance to any 'normal' exposure to Al in everyday life, and injection of Al citrate into the blood doesn't really tell you much at all about normal chronic exposure to Al via any route and including vaccination.

Using the same tracer ^{26}Al , Flarend and Hem [52,55] therefore carried out the only pharmacokinetic study of Al adjuvants and in an animal model. It should be noted that this study was initially considered as a preliminary study [53] but was not followed by any definitive study. The French National Academy of Medicine emphasizes that "this experimental work, unique to date, is used for the modeling of the pharmacokinetics of adjuvants" [4]. This unique reference study suffers from many weaknesses in its working hypotheses, its design, and the interpretation of its results.

3.1.1. An incorrect starting hypothesis

At the time of the study, the working hypothesis on how aluminum-based adjuvants work was that of Glennly [5], according to which Al adjuvant [initially Al potassium sulfate $\text{KAl}([\text{SO}_4]_2)$] formed a local deposit from which a gradual desorption of the vaccine antigen took place, at the origin of the observed adjuvant effect. The depot theory, as it is called, has recently been questioned [48], and now largely abandoned [58]. On the basis of this initial dogma, Stanley Hem, a chemist, had studied in vitro the dissolution kinetics of a dose of Al adjuvant (corresponding to 0.85 mg of Al) in 25 ml of a medium adjusted for citrate to mimic the concentration of Al chelating acid found in the interstitial fluid [59]. At pH 7.35 and ambient temperature, he observed that 55% of Al phosphate was dissolved at 12 h, compared to 0% for two commercial Al hydroxide adjuvants. By increasing the concentration of citrate by a factor $\times 100$ and raising the temperature to 37 °C, dissolution of 100% of the phosphate form was observed at 12 h compared with < 6% for the hydroxide forms. At 132 h (final study time), the dissolution of the hydroxide forms was only 7 to 10%. While mentioning the existence of different dissolution kinetics of Al phosphate and Al hydroxide forms in vitro, Flarend et al. have assumed as a starting point of their in vivo study that the two adjuvants injected into the tissue would be solubilized in contact with the organic chelating acids having an alpha-hydroxy-carboxylic acid group (citric acid, lactic acid and malic acid) present in the interstitial fluid.

This initial hypothesis is largely false in two aspects: the solubilization of Al hydroxide previously observed in vitro was nil in the presence of a physiological concentration of citrate and remained very low (6%) when citrate concentration was increased by 100 fold [59] and, above all, the authors were probably unaware of particles capture by immune cells. The fact that once injected into a tissue, agglomerates of adjuvant are rapidly captured by the cells of the innate immune system and thus rapidly taken away from the dissolving effect of the chelating agents present in the interstitial fluid was fully demonstrated several years later [21,50,60–62] but only occasionally documented prior to their study [63–65]. The authors implicitly recognized particles cellular uptake a few years later by showing the importance of phagocytosis in the adjuvant immunologic effect [66]. Incorrect starting hypothesis does not negate study results, of course, but phagocytosis obviously represents a critical factor that must be taken into account to interpret the results. One may argue that cells contain citric and malic acids as part of the citric-acid cycle and lactic acid from the anaerobic breakdown of glycogen, but the exact contribution, if any, of these intracellular chelating acids in adjuvant solubilization in vivo is

unknown. Moreover, another mean of mineral particle corrosion by the autophagy-lysosome machinery has been described [67], suggesting that aluminum-based adjuvant solubilization may largely depend on cell-specific genetically-driven mechanisms.

3.1.2. A study protocol with a limited and imperfect design

Flarend et al. [52] injected intramuscularly 0.85 mg of ^{26}Al as hydroxide or phosphate to rabbits.

- Only two rabbits were injected for each Al salt studied which appears to be a too small number of animals per condition required for reliable interpretation of data from biological experiments. Indeed, the experiments will show strong inter-individual variation of Al urinary elimination after Al-phosphate injection (see below and Fig. 1). Such inter individual variations were previously observed after intravenous Al injection in man [57];
- The study was conducted for a very limited period of 28 days: the team's previous in vitro results (see above) made it unlikely that Al hydroxide would be removed after such a short period of time [59];
- Al hydroxide used, manufactured by precipitation, differs from Al oxyhydroxide (Alhydrogel®) found in commercial vaccines [68]. [The same was true for Al phosphate that differed from Adju-Phos®].

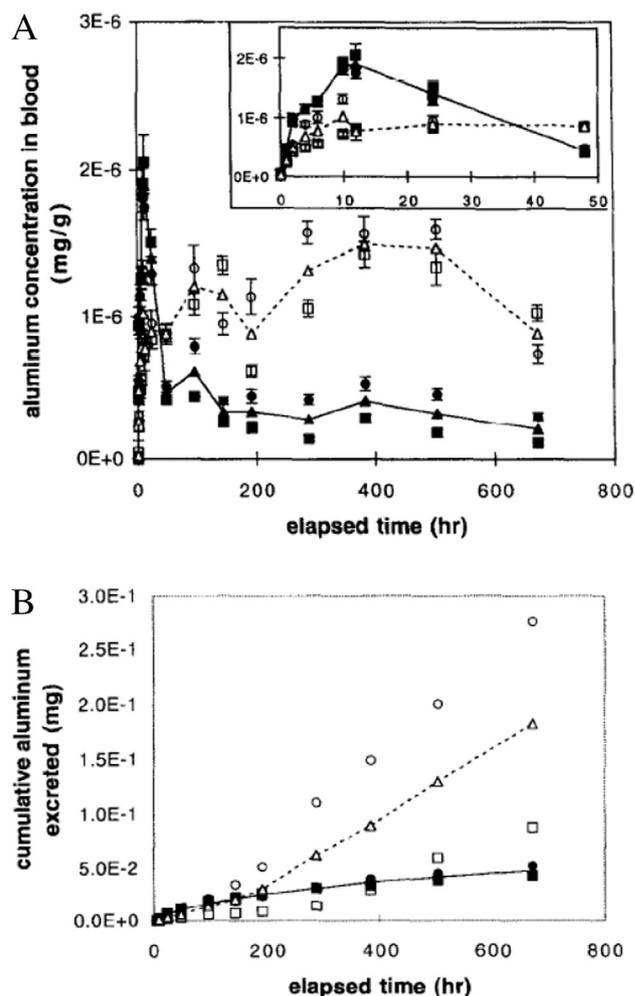


Fig. 1. A: This figure corresponds to the original Fig. 1 in Flarend et al. [52], showing plasma concentration kinetics of ^{26}Al after intramuscular injection of ^{26}Al hydroxide and ^{26}Al phosphate in rabbits.

B: This figure corresponds to the original Fig. 2 in Flarend et al. [52], showing cumulative urinary excretion of ^{26}Al after intramuscular injection of ^{26}Al hydroxide and ^{26}Al phosphate in rabbits. Reproduction of these figures with permission (Elsevier license # 4101280853249).

One possible option would have been to incubate the ^{26}Al for a long time with Alhydrogel® [or Adju-Phos®] and wait for the exchange between ^{27}Al and ^{26}Al in order to mark the official adjuvant.

3.1.3. Forgotten or destroyed target tissues

The lack of relevance of the organs removed at the end of the study to assess the biodistribution of ^{26}Al is striking:

- Muscle tissues at the injection site were not sampled making it impossible to determine the amount of adjuvant left at the injection site even though the study was based on “depot theory”;
- The sampled lymph nodes were intestinal lymph nodes and not the drainage ganglia of the injected area, whereas drainage of the adjuvant to the regional lymph nodes is a recognized route of systemic dissemination of adjuvant [50,69,70];
- The sampled bones (femur) were lost, which was unfortunate as bone is a known sink for circulating soluble aluminum, perhaps more useful than the kidney or other organs [71,72];
- The brains were sampled, though one of them was destroyed, the one which was taken from the animal with the highest blood content of ^{26}Al (animal injected with Al phosphate).

3.1.4. Initial plasma measurements contradictory to preliminary in vitro results

Flarend et al. [52] measured ^{26}Al in blood and urine during 28 days of the study and then in the post-mortem samples.

- Their first finding was the occurrence of an initial blood peak of ^{26}Al . Unexplainably, it was the hydroxide salt which induced the sharpest peak, the increase being noted from the first point (1 h), culminating at 10 h and ending at 48 h (Fig. 1A). The authors interpreted this initial peak as resulting from an early dissolution of Al hydroxide, which seems doubtful in the light of previous research which showed little solubilization of hydroxide adjuvant in vitro after 12 h [59]. On the other hand, the phosphate salt, which should solubilize more rapidly than the hydroxide, produced only a modest increase in plasma ^{26}Al , as evidenced by a higher area under the curve of a factor $\times 1.4$, for hydroxide within the first 48 h. One possible explanation for the higher plasma ^{26}Al in the rabbit receiving hydroxide adjuvant, which was not considered by the authors, is that nano or microparticulate Al hydroxide leaked from the injection site in blood due to needle damage at the injection site.
- From 48 h the plasma concentration of ^{26}Al plasma was higher for Al phosphate and remained higher than that for Al hydroxide thereafter (Fig. 1A). The authors do not comment on the existence of undulations in the plasma concentrations of ^{26}Al with peaks at 100 h and 400 h. These changes were seen in both adjuvants but were sharper for Al phosphate, which might suggest cyclical absorption phenomena, perhaps linked to cellular or tissue capture/release phenomena. At 28 days after the injections, the absorption, i.e. solubilization, of ^{26}Al from Al phosphate adjuvant is 3 times higher than that observed for Al hydroxide.
- At the end of the study, the authors insist on the absence of terminal phase in the curve of plasma concentrations, that is to say of terminal phase of blood absorption of ^{26}Al . Examination of the same curves by the Mitkus et al. [73] indicated that, in fact, the passage of ^{26}Al into the blood had initiated the terminal phase for Al phosphate and was already very close to zero for Al hydroxide on the 28th day of the study (Fig. 1A). For Al hydroxide, the plasma levels of ^{26}Al were very low since the 100th hour and absorption in blood further decreased from the 400th to the 700th hour, indicating an extremely low Al plasma passage after the initial peak observed from 0 to 48 h.

3.1.5. Statements on the elimination of adjuvants are not suggested by the results

There is a strong difference in urinary excretion of ^{26}Al between the

two adjuvants. At 28 days after the injections, 22% of the ^{26}Al originating from the phosphate adjuvant was eliminated in the urine, with substantial differences between the two studied rabbits (10–33%). At the same time, only 5.6% (5.0–6.2%) of the ^{26}Al originating from the hydroxide adjuvant was eliminated in the urine. The retention level of $> 94\%$ at 28 days observed for Al hydroxide is consistent with its expected low solubilization rate. Taking into account the initial blood peak, which interpretation in terms of solubilization is uncertain (see above), the authors calculate that only 17% of the ^{26}Al is absorbed from the hydroxide at 28 days of study (compared to 51% for phosphate). As a result, the distribution in the different tissues of ^{26}Al shows consistently higher tissue concentrations for the phosphate form (with a factor of about $\times 2.9$).

The distribution is similar for both adjuvants (kidney $>$ spleen $>$ liver $>$ heart $>$ lymph node $>$ brain), with reservations due to the lack of analyses of muscle at the injection site, the draining lymph nodes and bone. This tissue distribution of ^{26}Al is only valid for the short time of the study. This point is particularly important if one considers the possibility of a slow translocation of Al hydroxide from the injection site to the lymphoid organs [70] and the brain [69].

The authors correctly emphasize that urinary elimination of ^{26}Al persists in steady-state for both adjuvants at 28 days after the injections. However, the excreted cumulative dose study showed a clear increase over time in one of the rabbits for the phosphate adjuvant, while the slope was markedly lower for the second rabbit and quasi-flat for the aluminum hydroxide adjuvant (Fig. 1B). These results indicate that excretion may be slow for Al phosphate, and is very slow for Al hydroxide. The authors state that “the dissolution, absorption, distribution and elimination of Al adjuvants has been demonstrated” by their study. Rather than talking about the reassuring nature of these results [53], an inverse conclusion should have been made by the authors from a vaccine safety perspective, highlighting the low dissolution and low elimination of Al adjuvants, especially the hydroxide-based adjuvant, and the need for further long-term studies on a larger number of animals. The regulatory agencies themselves would have been well advised to order complementary toxicokinetic studies in order to avoid the propagation of hazardous information on the rapid elimination of Al adjuvants [54], especially after they had become aware of subsequent studies showing phagocytosis, intracellular persistence, distance migration and neurotoxicity of Al adjuvants [21,50,69,70].

3.2. Theoretical calculations which suggested the safety of multiple doses of Al vaccine administered to infants [73,74]

Two studies compared the theoretical impact of dietary Al and vaccine-derived Al in infants [73,74]. The principle of the two studies is similar: these are theoretical calculations based on the intake and excretion of aluminum from birth to 12 months. The calculated accumulation of aluminum is compared to the safety level determined for the oral route by the Agency for Toxic Substances and Disease Registry in Atlanta (ATSDR). The ATSDR defines a minimal risk level (MRL) that takes into account the risk of neurotoxicity of aluminum administered orally. This oral MRL is fixed from animal experiments extrapolated to humans using correction factors. The “reassuring” results of these two theoretical studies have been a strong argument in favor of the safety of Al adjuvants [4,54]. In addition, a single direct study was conducted in human infants, on the short term (24 h) in preterm infants [75].

3.2.1. Study by Keith et al. [74]: too high “safety” threshold, erroneous absorption model, and key organ's immaturity were not considered

3.2.1.1. Description of the study. Keith et al. [74] estimated the accumulation of aluminum in the body according to the age and weight of children from 0 to 12 months. Dietary accumulation (breastfeeding and/or artificial feeding) was calculated by taking into account an intestinal absorption factor of 0.78%. Contribution from vaccines, i.e. 7 injections administered at 0, 2, 4, 6 and 12 months (3

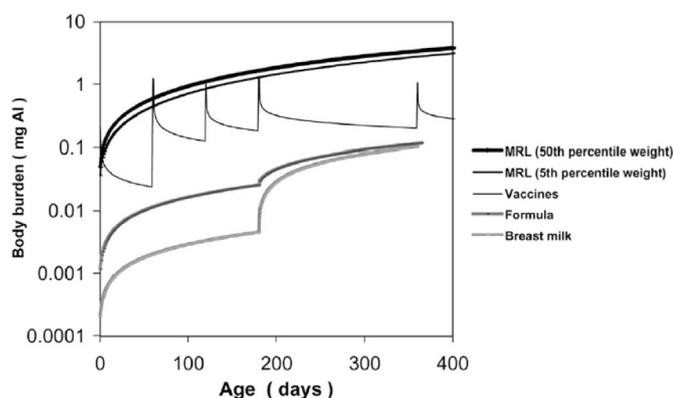


Fig. 2. Figure from Keith et al. [74] assessing Al body burden contributions from diet and vaccines in infants. Safety limit curve integrates the oral Minimum Risk Level (MRL) based on an experimental Non Observable Adverse Effect Level (NOAEL) value of 62 mg Al/kg/d, and the body weight of US kids. In case of an immediate absorption of 100% of vaccine Al, there is a transient overstep of the safety limit by the Al vaccine at 2 months, and peaks reaching the limit at 4 and 6 months. Reproduction of this figure with permission (Elsevier license # 4101280948254).

anti-hepatitis B and 4 DTaP (diphtheria-tetanus-acellular pertussis)) was calculated assuming that injected Al is immediately absorbed at 100% and that the toxicokinetic profile is the one described and modeled by Priest for the soluble ^{26}Al intra-venously injected in man [56].

These intakes were compared with a “safety” profile taking into account the 0–12 month weight increase and an MRL of 2 mg Al/kg/day [76]. This MRL was defined from an earlier study of Golub et al. [77] who had studied the motor activity of mice subjected to a feed containing Al lactate. In these mice the Non-Observable Adverse Effect Level (NOAEL) was 62 mg Al/kg/d, corrected by a factor $\times 30$ [extrapolation factor $\times 3$ from mouse to human and factor $\times 10$ for inter-individual variability], which produced an oral MRL of 2 mg Al/kg/d [75]. The study by Keith et al. [74] showed that accumulation of Al from vaccines was about twice that of dietary intake but remained largely below the MRL curve. However, the authors pointed out that in their model, vaccines in the vaccine schedule produced peaks at each injection, and the one from the 2nd month briefly exceeded the MRL curve and those of the 4th and 6th month were just at the limit of this curve (Fig. 2).

3.2.1.2. Critique of the study. The limitations and methodological imperfections of the model of Keith et al. [74] justified the subsequent study of Mitkus et al. [73]. Mitkus et al. felt that several limitations in the Keith work, detailed below, deserved a novel study:

- Subsequent amplification of the pediatric vaccine schedule recommended in the USA between the ages of 0 and 12 months; 3 Al-adjuncted vaccines (7 injections) were added to the hepatitis B and DTaP vaccines, including vaccines against *Haemophilus influenzae*, *Pneumococcus* and hepatitis A. In 2016, 17 Al-adjuncted injections were recommended by the CDC for infants between the age of 0 and 18 months (Table 2) [78]. This number is a maximum because of the possible use of various multivalent vaccines;
- Subsequent lowering of the safety level for Al, with the oral MRL decreasing from 2 to 1 mg Al/kg/d in 2008 [78];
- Failure to take into account the immaturity of the glomerular filtration function in the infant which may affect the removal of aluminum [73]; it should be noted that the issue of the blood-brain barrier has not been taken into account even though the development of the nervous system is notoriously sensitive to toxic exposures [80]. The issue of blood-brain barrier immaturity is an important issue in the potential toxicity of Al adjuvants. In its report, the French National Academy of Pharmacy [13] considers that “the blood-brain barrier, which is incompletely formed in the pre-natal

Table 2

Recommended immunization schedule for infants aged 0 through 18 months [CDC 2016].

Age [months]	Birth	2	4	6	12	15	18
Vaccine							
Hepatitis B ^a	x	x		x			
Rotavirus			x	x	x		
DTaP (diphtheria, tetanus, acellular pertussis) ^a		x	x	x			x
Hib (<i>Haemophilus influenzae</i>) ^a			x	x	x		x
Pneumococcus ^a		x	x	x			x
PVI (inactivated poliovirus)		x	x	x			
Influenza						x	x
Measles-mumps-rubella						x	
Small pox						x	
Hepatitis A ^a						x	x

^a Al-containing vaccine (Al hydroxide and/or Al phosphate).

and post-natal stages, is more permeable to toxic substances. In addition, the brain is more perfused between 6 and 13 years because of its increased needs for maturation. [...] Experimental toxicological studies conducted in juvenile animals [...] are mandatory since epidemiological studies in children [...] are hardly feasible” [13].

- Necessary updating of the weight curve of American children [72];
- Improvement in 2004 of the mathematical modeling of the retention of IV-injected ^{26}Al in humans, now comprising 3 phases of absorption with respective Al half-lives of 1.4, 40 and 1727 days [81];
- And above all, taking into account the results of Flarend et al. [52] showing that Al absorption (solubilization) from the adjuvants can under no circumstances be considered as 100% immediately after injection.

3.2.2. The study by Mitkus et al. [73]: “safety” threshold still too high, nano/microparticulate Al not considered as potentially noxious

3.2.2.1. Description of the study. In this study, Mitkus et al. revisited Keith's methodology, taking into account all limitations listed above. At first, they did not take into account Flarend's pharmacokinetic results, confirming Keith's paper assumption that, if plasma uptake of Al from vaccines would immediately represent 100% of the dose -an hypothesis that maximizes the body burden-, there would be a transient crossing of the calculated security threshold at 2 months and a peak at the limit of the threshold at 4 months. Then Mitkus et al. took into account the slow absorption (solubilization) of Al from adjuvants shown by Flarend, and, in so-doing, found a seemingly high safety margin. To build their model, Mitkus et al. reasoned as follows: since the blood absorption of aluminum was 51% for phosphate adjuvant at 28 days after the injections in the Flarend study, it would take 28 more days to absorb the whole injected dose of adjuvant (total 56 days). Similarly since blood absorption of Al was 17% for Al hydroxide at 28 days, complete absorption would take 137 additional days (total 165 days). The calculated cumulative amount of aluminum absorbed from vaccines was significantly higher than the dietary Al uptake (factor $\times 2$) but remained below the safety level for Al phosphate, and very largely below for Al hydroxide (Fig. 3). The author's conclusion is that the Al from vaccines is unlikely to have a significant influence on Al body burden of the infant's organism, implying a good safety of Al adjuvants from 0 to 12 months.

3.2.2.2. Mitkus' study suffers from a number of important biases.

- An inappropriate oral MRL was used to define the safety curve. The ingested Al was said to cross the intestinal barrier in its ionic form [74]. On the other hand, the adjuvants are nanoparticles aggregated in microparticles administered directly beyond the skin barrier. However, particulate toxicology involves many other parameters than the dose. In particular, the particle surface increases exponentially as the particle size decreases [and the number of

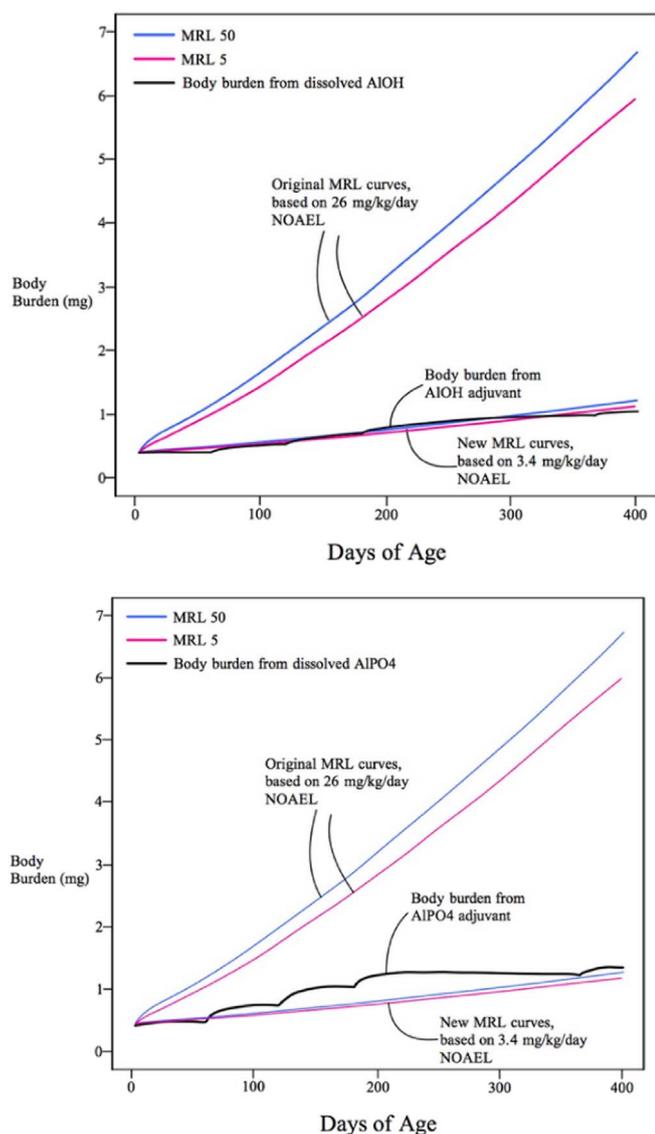


Fig. 3. These curves are derived from those of Mitkus et al. [73], in infants. On both panels oral Minimum Risk Level (MRL) curves (the two upper curves) of the original article were based on an experimental Non Observable Adverse Effect Level (NOAE) of 26 mg/kg/d and integrated actualized American child body weight curves; revised MRL curves (the two lower curves) are based on an actualized NOAE of 3.4 mg Al/kg/d [88,91]. Al absorbed from both Al hydroxide and Al phosphate according to Flarend et al. [52] absorption rates, show over-step of the safety limit.

particles increases] for a given mass of material [82]. In its particulate form, Al is rapidly captured and then transported at a distance by immune cells [21,50,69,70]. The comparison of the chemical toxicity of Al ions, such as those absorbed at the intestinal level, and the particulate toxicity of Al salts injected IM is therefore nonsense [83]. This is evidenced by the atypical dose-response curve of the neurotoxic effects of Al hydroxide, with cerebral transfer of aluminum and a clinical effect selectively observed for low dose, which approximates those described in particulate toxicology [84]. Strictly speaking, MRL used for vaccine risk modeling should be defined on the basis of animal experiments carried out with Al adjuvants, monitored for their particle parameters to be in accordance with those of the vaccines, and injected IM, rather than studies with soluble forms of Al [chloride or lactate] added to food or drinking water.

- Based on experimental data, oral MRL sets the safety curve too high. The MRL of 1 mg/kg/d [79] was determined based on a NOAE of

26 mg/kg/day observed in mice in 2001 [85]. However, there are numerous reports of neurotoxic effects in mice and rats, confirmed by coherent neurobiological alterations, for oral doses of Al much < 26 mg/kg/d: 6 mg/kg/d reported in 1993 [86], 5.6 mg/kg/d reported in 2008 and 2009 [87,88], 10 mg/kg/d reported in 2016 [89], 3.4 mg/kg/d reported in 2016 and 2017 [90,91], and even 1.5 mg/kg/d reported in 2017 [92]. By using the “official” oral MRL, Mitkus therefore set the safety curve at a much higher level. This level was overestimated by a factor of up to 17.3 (i.e. 26/1.5) when the most recent study was taken into account. It should be noted that the 1.5 mg/kg/day reported is not even a NOAE since effects have been documented at this dose [92]. Fig. 3 shows that even if one uses higher experimental NOAE levels for calculation, e.g. 3.4 mg/kg/d, the safety limit is reached (hydroxide) or overstepped (phosphate) by Al from vaccine adjuvants. Under these conditions, the safety of Al adjuvants in infants cannot be guaranteed without doubts on the basis of the Mitkus study.

- Potential toxicity of particulate Al was not considered. Like Flarend before him [52], Mitkus et al. seemingly considered that only the soluble Al has toxic potential. His estimation of the duration of complete translocation of Al from the injected site to blood (< 2 months for the phosphate, 5.5 months for the hydroxide) is based on a simplistic calculation (see above) not taking into account that Flarend's curves suggest that the termination of Al translocation to plasma is either underway (phosphate) or nearly achieved (hydroxide) on the 28th day (see above). The corollary of this oversimplistic calculation is an underestimation of the bio-persistence time of Al in particulate form. Histological studies carried out after IM injection of Al hydroxide showed that particulate Al and the granulomas it induces, are still detectable in the injected muscle after months in animal studies [60,61] and several years (up to 12 years) in adult patients with chronic post-vaccine fatigue syndrome [93]. Although genetic factors might explain the low intracellular solubilization of Al hydroxide in susceptible individuals [93], the Mitkus underestimation of the stability towards dissolution of aluminum adjuvants is certain and significant.

Another limitation of the Mitkus study is that it does not take into account that the adjuvant can migrate away from the muscle in its particulate form. Experimental studies have shown that the long intracellular bio-persistence of Al hydroxide relates to particles observed at the injection site as well as those transported to distant organs [70]. In mice Al hydroxide particles are indeed transported by cells of monocytic lineage, first to the draining lymph nodes and then, probably via the thoracic duct, to the bloodstream, then reaching distant organs such as the spleen or even the brain, where slow and delayed accumulation can be observed in microglial cells and neurons [50,69]. After a single IM injection, cerebral penetration of the particles is low but increases considerably under the influence of Monocyte Chemoattractant Protein-1/Chemokine Ligand (MCP-1/CCL2) signaling, and is accompanied by cellular expression of Interleukin IL1beta, an expected effect of Al adjuvant-induced activation of the inflammasome [69]. Finally, it should be noted that neurotoxic effects have been observed in mice injected with doses of Al hydroxide reproducing an equivalent of the American vaccination schedule from age 0 to 18 months [94]. Considering soluble Al only, Mitkus thought that “long-term storage depot (of Al solubilized from the injected site), is likely to be skeletal and not a more sensitive soft organ system is reassuring”. This reassuring assumption did not take into account the fate of particulate Al. In the same way, a recent study performed in premature infants vaccinated at the age of 2 months [75], only focused on soluble Al detectable in body fluids: the authors curiously felt “reassuring” the fact that they did not notice elevation of Al in serum and urine 24 h after administration of vaccines containing a total dose of 1200 µg Al (about 200 µg/kg) [75]. The absence of both detectable absorption and rapid elimination of Al from adjuvants rather represents a legitimate reason

of concern, since, as a corollary, it likely indicates systemic persistence of immunostimulating and neurotoxic Al particles translocated to lymphoid organs and potentially reaching the brain [70,84].

4. Conclusion

The glorious history of vaccines was largely built on an empirical basis during the last century. This was the case for the first-generation aluminum-based adjuvants which, nevertheless, proved to be very useful since their introduction in 1926. These adjuvants are still intended to be administered to billions of individuals over the next years, because of a massive expansion of vaccine prevention strategies announced worldwide [2]. In this context, given their serious conceptual and methodological weaknesses, the 3 available toxico-kinetic studies objectively constitute insufficient bases to guarantee the absolute safety of aluminum adjuvants administered at very large scale, in particular over the long term. Vaccinology in the 21st century is a modern and strong science. As such, it cannot simply rely on its past successes, and make no effort to finely understand the *in vivo* fate of aluminum adjuvants, with the risk of losing the necessary confidence of populations which became extremely sensitive to every dimensions of global health. It seems to us highly mandatory to conduct new toxico-kinetic experiments, including long-term studies, under the tight control of health authorities, in order to ensure a maximum level of safety of both classical and new generation aluminum adjuvants used in vaccines.

Disclosure

The authors report no conflicts of interest.

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