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Non-linear dose-response of aluminium hydroxide adjuvant particles: Selective low dose neurotoxicity

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ABSTRACT

Aluminium (Al) oxyhydroxide (Alhydrogel®), the main adjuvant licensed for human and animal vaccines, consists of primary nanoparticles that spontaneously agglomerate. Concerns about its safety emerged following recognition of its unexpectedly long-lasting biopersistence within immune cells in some individuals, and reports of chronic fatigue syndrome, cognitive dysfunction, myalgia, dysautonomia and autoimmune/inflammatory features temporally linked to multiple Al-containing vaccine administrations. Mouse experiments have documented its capture and slow transportation by monocyte-lineage cells from the injected muscle to lymphoid organs and eventually the brain. The present study aimed at evaluating mouse brain function and Al concentration 180 days after injection of various doses of Alhydrogel® (200, 400 and 800 μg Al/kg of body weight) in the *tibialis anterior* muscle in adult female CD1 mice. Cognitive and motor performances were assessed by 8 validated tests, microglial activation by Iba-1 immunohistochemistry, and Al level by graphite furnace atomic absorption spectroscopy.

An unusual neuro-toxicological pattern limited to a low dose of Alhydrogel® was observed. Neurobehavioural changes, including decreased activity levels and altered anxiety-like behaviour, were observed compared to controls in animals exposed to 200 μg Al/kg but not at 400 and 800 μg Al/kg. Consistently, microglial number appeared increased in the ventral forebrain of the 200 μg Al/kg group. Cerebral Al levels were selectively increased in animals exposed to the lowest dose, while muscle granulomas had almost completely disappeared at 6 months in these animals.

We conclude that Alhydrogel® injected at low dose in mouse muscle may selectively induce long-term Al cerebral accumulation and neurotoxic effects. To explain this unexpected result, an avenue that could be explored in the future relates to the adjuvant size since the injected suspensions corresponding to the lowest dose, but not to the highest doses, exclusively contained small agglomerates in the bacteria-size range known to favour capture and, presumably, transportation by monocyte-lineage cells. In any event, the view that Alhydrogel® neurotoxicity obeys "the dose makes the poison" rule of classical chemical toxicity appears overly simplistic.

1. Introduction

Many severe infectious diseases can be prevented and some of them have been eradicated by vaccines. Commonly used vaccines are generally well tolerated and considered safe by regulatory agencies. However, as other effective medical compounds, vaccines may occasionally cause adverse effects. In particular, a condition

Abbreviations: Al, aluminium; dLNs, draining lymph nodes; im, intra-muscular; MMF, macrophagic myofasciitis; NOR, novel object recognition test; PFA, paraformaldehyde.

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manifesting by the combination of myalgia, arthralgia, chronic fatigue, cognitive dysfunction, dysautonomia and autoimmunity has been temporally linked to aluminium adjuvant-containing vaccine administration, called Macrophagic Myofasciitis (MMF) (Gherardi and Authier, 2003; Authier et al., 2003; Exley et al., 2009; Rosenblum et al., 2011; Santiago et al., 2014; Brinth et al., 2015; Palmieri et al., 2016).

Although no consensus has been reached so far on a cause-to-effect relationship, environmental aluminium has long been suspected to act as a co-factor of several chronic neurological diseases (Van Rensburg et al., 2001; De Sole et al., 2013; Exley 2013, 2014) and the idea has emerged that aluminium adjuvants may be insidiously unsafe over the long-term in some predisposed individuals (reviewed in Tomljenovic and Shaw, 2011; Gherardi et al., 2015). Among aluminium salts used in vaccines, crystalline Al hydroxide or oxyhydroxide (Alhydrogel®) is the more widely used and is found in vaccines against tetanus, hepatitis A, hepatitis B, *Haemophilus influenzae* B, pneumococcal and meningococcal infections, and anthrax (Gherardi et al., 2015). This adjuvant consists of primary particles in the nano-sized range spontaneously forming micron-sized agglomerates (Eidi et al., 2015).

Although aluminium salts have been added to vaccines since 1926 (Glenny et al., 1926), exact mechanisms underlying their immuno-potentiating effects remain incompletely understood (Exley et al., 2010). Previous studies from our laboratory have shown that alum particles, as other poorly degradable particles, may not stay entirely localized in the injected tissue in mice, but can disseminate within phagocytic cells to regional lymph nodes and then to more distant sites and to the brain (Khan et al., 2013; Crépeaux et al., 2015; Eidi et al., 2015). In contrast to a previous belief, alum is characterized by striking biopersistence within immune cells in both the injected muscle, and the draining lymph nodes (dLNs) and spleen, where it may be found in conspicuous quantities 9 months after injection (Crépeaux et al., 2015). In humans, long term biopersistence of aluminium hydroxide within innate immune cells causes a specific lesion at site of previous immunization, called MMF, that may be detected up to >12 years after the last vaccine injection (Gherardi et al., 2001) in patients with a clinical condition now designated as ASIA 'Autoimmune/inflammatory syndrome induced by adjuvants' (Shoenfeld and Agmon-Levin, 2011).

The potential impact of aluminium adjuvant on the nervous system has been studied in mouse models. Alhydrogel® adjuvant, dosed at 100 μg Al/kg and subcutaneously injected in CD1 mice induced motor deficits and cognitive alterations associated with motor neuron death and a significant increase (350%) of reactive astrocytes indicative of an inflammatory process (Petrik et al., 2007). Although no motor neuron death was observed at the dose of 300 μg Al/kg, both microglial and astroglial reactions were observed in the spinal cord and were associated with altered motor and cognitive functions in CD1 mice (Shaw and Petrik, 2009).

In the same way, a neuro-inflammatory/degenerative syndrome has been described in sheep after repeated administrations of alum-containing vaccines (Luján et al., 2013), and impairment of neurocognitive functions and brain gliosis were reported in a murine model of systemic lupus erythematosus-like disease following intramuscular injection of Al hydroxide or vaccine against the hepatitis B virus (Agmon-Levin et al., 2014).

Previous in vivo aluminium adjuvant neurotoxicological studies did not include dose-response analyses. However, several reports studying neurotoxicity of soluble aluminium compounds administered by the oral route (Al chloride, Al nitrate, Al ammonium sulfate) to rodents showed a non-linear biphasic response on acetyl-cholinesterase activity (Kumar, 1998), dopamine turnover (Tsunoda and Sharma, 1999), nitric oxide synthase expression (Kim, 2003), and behavioural performances (Roig et al., 2006). Poorly understood biphasic Al effects were also observed in vitro: cell cultures showing increased cell growth at low concentrations and diminished cell growth at high concentrations (Exley and Birchall, 1992). Similar unusual observations were made in studies of hippocampal long-term potentiation (Platt et al., 1995), and neuronal cell death in NSC-34 neuron-like cells (Eidi et al., 2015).

The present dose-response study was designed to evaluate long-term aluminium hydroxide neurotoxicity by assessing mouse behaviour, aluminium cerebral concentrations and microglial changes in CD1 mice 180 days after intramuscular injections of Alhydrogel®. Strikingly, the lower dose selectively induced neurobehavioural changes, cerebral aluminium level increases and microglial activation.

2. Materials and methods

2.1. Alhydrogel® doses

Animals were injected with Alhydrogel® adjuvant (InvivoGen), the characteristics of which have been previously determined in terms of size and positive zeta potential (Eidi et al., 2015). Doses were calculated by reference to medical histories of MMF patients who received a median of 4 doses of an Al-containing vaccine within the 10 years prior to their diagnosis (Gherardi et al., 2001). A 60-kg woman (MMF affects mainly women) injected with 1 dose of HBV ENGERIX® vaccine (GSK laboratories, France) receives 500 μg of Al, i.e. 8.3 μg Al/kg of body weight. Extrapolating mouse to human dosage is a challenging issue. Although a firm scientific basis for allometric conversion is still lacking, we used an allometry calculation based on body surface area that reflects the metabolic rate to determine the human equivalent dose per Kg. This ×12.3 allometric conversion factor from human to mouse (Sharma and McNeill, 2009) is easy to apply, and has been recommended to us by toxicologists of the French drug agency (AFFSAPS). Conversion resulted in an approximate of 100 μg Al/kg mouse body weight for one human dose. Four groups were used: control group (phosphate buffered saline (PBS) vehicle: Phosphate 0.1 M; NaCl 0.9%; pH 7,4); Alhydrogel® groups at the doses of 200, 400 or 800 μg Al/kg, in 3 injections of Alhydrogel in 20 μL PBS with a four-day interval. The animals thus received the mouse equivalent of 2, 4 and 8 human doses of Al-containing vaccine.

2.2. Animals

40 female CD1 mice, weighing 25–30 g (7 week old), were obtained from Charles Rivers Laboratories (France). Upon arrival, the females were housed at 5 animals per cage. Animals were maintained under a 12 h light cycle (8.00: 20.00), at a constant temperature (22 ± 2 °C) and a relative humidity of 55 ± 10%. Mice were given ad libitum access to food and water. After a 1-week period for acclimatization, 8-week old females were separated in 4 experimental groups of 10 animals, and 20 μL im injections were made in the left *tibialis anterior*, with a 4-day interval between each injection.

At the end of the behavioural tests, 5 animals per group were sacrificed with an overdose of pentobarbital and transcardially perfused with PBS followed by ice-cold 4% paraformaldehyde (PFA) in PBS. Brains were collected for histological examination, post-fixed in PFA for 4 h at 4 °C and immersed overnight in a 30% sucrose/PBS solution, then frozen and stored at −80 °C until sectioning. Whole brains were serially cut into 40 μm-thick coronal cryosections stored at −20 °C until use.

The other 5 animals per group were sacrificed with an overdose of pentobarbital. Brains were retrieved, quickly frozen in isopentane and kept at −80 °C for subsequent determination of Al levels.

All the experiments on animals were performed in respect to the guidelines provided by the European Union (Directive 2010/63/EU).

2.3. Behavioural and motor testing

A battery of 8 behavioural or physical tests was performed in the 4 experimental groups (n = 10 mice/group) 180 days after the third injection. Tests were chosen in order to assess locomotor activity in the open-field (Walsh and Cummins, 1976), level of anxiety in the O-maze (Shepherd et al., 1994; Coutellier et al., 2009), short-term memory in the novel object recognition test (Ennaceur and Delacour, 1988; Dudchenko, 2004; Ennaceur, 2010; Moore et al., 2013), muscular strength in the wire mesh hang (Kondziela, 1964) and the grip strength tests (Maurissen et al., 2003), locomotor coordination in the rotarod test (Pratte et al., 2011), depression in the tail suspension test (Steru et al., 1985), and pain sensitivity in the hot plate test (Espejo and Mir, 1993).

All the tests were performed under white light <100 Lux between 9 a.m. and 1 p.m. They were video-recorded and all the variables were analyzed by the same experimenter, using ViewPoint Life Sciences Inc software (Canada).

The animals were transferred to the behavioural testing room 30 min prior to beginning of test in order to let the animal adapt to the test room conditions. Between each animal, the apparatus was cleaned with a 30% ethanol solution. At the end of a whole testing session, mice were sacrificed and samples were retrieved.

2.3.1. Open-field

The general locomotor activity was assessed by the open-field test (Walsh and Cummins, 1976). The apparatus was made of a square open-field arena (42 cm side × 25 cm high walls) with the floor divided into 3 distinct areas: the peripheral, the medium and the central areas. At the beginning of the test, the mouse was placed in the center of the central area, and was let free to explore for 5 min. During this period the total distance and the distance and time spent in each of the three areas and the number of rearing, were recorded.

2.3.2. Elevated O-maze

The level of animal anxiety was assessed by the elevated O-maze test (Shepherd et al., 1994), with the advantage of the lack of the ambiguous central square compared to the traditional plus-maze (Coutellier et al., 2009). The maze was elevated to a 70 cm height, with 2 open (50 × 10 cm) and 2 closed (50 × 10 × 40 cm) arms. Arms of the same type were opposite to each other. Each mouse was tested within a 5-min test session. At the beginning, a mouse was placed individually in one of the closed arms, and was allowed to freely explore the maze. The time spent in closed and open arms, latency time to exit the closed arm for the first time, and the number of head-dippings and rearings were recorded.

2.3.3. Novel object recognition test

The novel object recognition test (NOR) was first proposed by Ennaceur and Delacour in 1988. This test is based on the spontaneous behaviour of rodents to interact more with a novel object than with a familiar one because of their inherent preference for novelty. Thus, in this test, rodents must be able to remember the previously encountered familiar object to determine which object is “novel” during the test trial (Moore et al., 2013).

The NOR task can be configured to cover various aspects and types of memory, including working memory (Dudchenko, 2004; Ennaceur, 2010).

The apparatus consisted of a square chamber (40 × 40 × 25 cm) and a digital camera was used to record behaviour videos. Videos were analyzed and the time spent by mice exploring each object was measured. The test consisted of four sessions: habituation to the field (10 min, day 1), habituation to objects (5 min, day 1), familiarization phase with 2 identical objects (5 min, day 2), test 1 h later (5 min, day 2), with one familiar and one novel object. The novel objects were different in shape and colour but similar in size. The interaction of mouse with both objects (familiar and novel) was recorded for 5 min and percent discrimination index was calculated to determine memory performance as follow:

Discrimination index = exploration time with novel object/(exploration time with familiar object + novel object) × 100.

Exploration of an object is defined as the orientation of animal’s snout toward the object, sniffing or touching with snout, while running around the object, sitting or climbing on it was not recorded as exploration (Antunes and Biala, 2012).

2.3.4. Wire mesh hang test

The hang wire mesh test was designed to test muscle strength using all four limbs (Kondziela, 1964). The inverted screen is a 43 cm square of wire mesh consisting of 12 mm squares of 1 mm diameter wire. The time during which the animals were able to sustain their weight holding onto the metal rail suspended in midair above the surface of soft bedding material was recorded for a 5 min-maximum time. Each mouse was subjected to three trials and the best performance was retained. Mouse body weight was considered, because this variable can influence performance.

2.3.5. Grip strength test

The rodent grip strength test was developed to measure muscular strength (Maurissen et al., 2003). The apparatus (Bio-GS3, Bioseb, France) consists of a grasping device or platform (i.e. grid and T-bar) that is connected to a load cell. The test measurement is conducted by allowing the animal to grasp the device and then having the experimenter pull it away until its grip is broken. The maximal force achieved by the animal was recorded for two types of measurements: forelimb measurement and forelimb and hindlimb measurement. Five such trials for the forelimbs and five others for the four limbs were performed and both best performances were kept.

2.3.6. Accelerating rotarod

Motor coordination and balance were tested using an accelerating rotarod (LE8200, Bioseb, France) consisting of a 3 cm diameter drum (15 cm above the base), divided with flanges into five lanes (Pratte et al., 2011). The apparatus is electronically controlled and evenly increases the speed of the bar from 4 to 40 rpm over a 5-min session. The mice were placed on the rod body orientation opposite to beam movement in the longitudinal axis, so that forward locomotion was necessary to avoid a fall. The mice were acclimated and trained on a morning session, and then they were given five successive trials on the afternoon. The best trial (longest latency to fall) for each mouse was retained. Since body weight may affect performance, mouse weight was considered in the score determination.

2.3.7. Tail suspension test

The method is based on the observation that a mouse suspended by the tail shows alternate periods of agitation characterized by intense motor activity and expense of energy, and waiting-behaviour with immobility and energy saving (Steru et al., 1985).

For these experiments, the mouse was hung on a hook by an adhesive tape placed 20 mm from the extremity of its tail. Mice were both acoustically and visually isolated. Each mouse was

suspended by its tail for 5 min, allowing the ventral surface and front and hind limbs to be video-recorded using a digital camera facing the test box. Total immobility time and latency time to be immobile were measured during the entire 5 min test period. Immobility was defined as the absence of initiated movements, and included passive waving of the body. Times were scored manually by observer watching the video. Each mouse was tested only once. Mouse body weight was considered in the score determination.

2.3.8. Hot plate test

The hot plate test is a behavioural model of nociception in which mice display several noxious-evoked patterns as well as exploratory and self-care responses (Espejo and Mir, 1993). The animals were individually placed on a preheated 50 °C hotplate (LE7406 Bioseb, France). An open-ended cylindrical Plexiglas tube with a 20 cm diameter and a 25 cm height was placed on top of the hot plate to prevent the mice from escaping but leaving their paws exposed to the hot plate. The time from placing the animals on the hot plate to the time of the first paw lick, the first rearing and the first jump were measured with a stopwatch. To prevent issue damage, the mice were removed from the hot plate after 3 min regardless of their response. Mice were observed only once.

2.4. Microglia immunohistochemistry

Analyses were carried out on 3 brains per group. Brain sections were incubated with primary antibody Anti-Iba1 (goat ab5076, AbCam Paris, France, 1/2000 in PBS with 1% BSA) overnight at 4 °C. Then sections were incubated with secondary biotinylated rabbit anti-goat antibody (1/200, Vector Laboratories, Paris, France) for 2 h at room temperature. Labeling was determined using the chromogenic diaminobenzidine (DAB) method.

Microscopy: Brain sections were viewed with a Zeiss AxioPlan (Carl ZeissCanada Limited, Toronto, ON, Canada) microscope at 20× magnification. Images were captured using Zen2012 software. Microglia cell density and cell body area were measured in 4 regions mapped by reference to the Paxinos mouse brain atlas (Paxinos and Franklin, 2001): ventral forebrain, inferior colliculus and visual and motor cortex. Determinations were done on selected areas (mean area of 175,000 μm^2) in 3 animals per group, by at least 2 of us, blinded for the identity of the group.

2.5. Brain Al analysis

Analyses were carried out on 5 brains per group (groups PBS, Alhydrogel® 200, 400, 800 μg Al/kg) 180 days following injection, according to the published method of House et al. (2012) and as described in our previous study (Crépeaux et al., 2015). Briefly, Al concentrations were determined by TH GFAAS in half brains dried to a constant weight at 37 °C and digested in a microwave (MARS Xpress CEM Microwave Technology Ltd) in a mixture of 1 mL 15.8 M HNO_3 (Fischer Analytical Grade) and 1 mL of 30% w/v H_2O_2 (BDH Aristar Grade). Digests were clear and colourless or light yellow with no visible precipitate or fatty residue. Upon cooling each digest was diluted to a total volume of 5 mL with ultrapure water. Total Al was measured immediately post digestion using an AAnalyst 600 atomic absorption spectrometer with a transversely heated graphite atomizer (THGA) and longitudinal Zeeman-effect background corrector and an AS-800 autosampler with WinLab32 software (Perkin Elmer, UK). Standard THGA pyrolitically-coated graphite tubes with integrated L'Vovplatform (Perkin Elmer, UK) were used. The Zeeman background corrected peak area of the atomic absorption signal was used for the determinations.

Results were expressed as μg Al/g tissue dry weight. Each determination was the arithmetic mean of a triplicate analysis.

2.6. Muscle analysis

Analyses were carried out on 3 muscles per group (groups PBS, Alhydrogel® 200, 400, 800 μg Al/kg) 180 days following injection. Serial muscle tissue sections of 10 μm were successively deposited on 30 different Superfrost®-plus slides in order to obtain 30 identical series. For each animal one slide containing 20 representative longitudinal sections was used for haematoxylin-eosin staining, and two alternate slides were treated for Morin staining and CD11b immunostaining respectively.

- Immunostaining was done using commercial primary antibody routinely used in the lab, raised against CD11b (1/50, AbD Serotec, MCA711, Oxford, UK). The labeling was made with Cyanine 3 AffiniPure F(ab')$_2$ Fragment Donkey Anti-Rat (1/200, Jackson ImmunoResearch laboratory INC, Suffolk, UK).
- Al was stained with Morin (M4008-2 G, Sigma-Aldrich, Saint-Quentin-Fallavier, France) that was dissolved in a solution consisting of 0.5% acetic acid in 85% ethanol. Formation of a fluorescent complex with Al was detected under a 420 nm excitation wavelength as an intense green fluorescence with a characteristic 520 nm emission.
- Conventional microscopy was done using Carl Zeiss photonic and fluorescence microscopes.
- The presence of a muscle granuloma was semi-quantitatively assessed at magnification ×20, and quoted as: 0 (no or virtually no inflammatory cell), + (1 to 3 small granulomas), ++ (>3 small granulomas), +++ (>3 large granulomas).

2.7. Statistical analysis

Normality distribution of data was first analyzed by Shapiro-Wilk test, and then parametric or non-parametric tests were decided according to p values of Shapiro-Wilk test, i.e. parametric

Table 1
Effects of different doses of Alhydrogel® on motor activity and anxiety assessed in the open-field.

Open field	Control			Alhydrogel® 200 μg/kg			Alhydrogel® 400 μg/kg			Alhydrogel® 800 μg/kg			ANOVA	
	mean	±	sem	mean	±	sem	mean	±	sem	mean	±	sem	$F_{(3,39)}$	*p*
Total distance (cm)	2401.01	±	300.62	**1303.48**	±	**213.04***	2181.90	±	166.76	2622.46	±	205.96	4.220	$p < 0.05$
Distance in central area (cm)	236.34	±	35.96	228.01	±	44.92	163.33	±	31.02	238.50	±	38.89	0.831	n.s.
Distance in intermediate area (cm)	677.41	±	108.29	431.85	±	86.21	459.20	±	64.02	811.68	±	76.32	3.205	$p < 0.05$
Distance in peripheral area (cm)	1487.26	±	173.89	**643.61**	±	**142.06***	1530.20	±	106.15	1572.29	±	200.96	6.025	$p < 0.01$
Time spent in central area (s)	25.30	±	5.17	**62.34**	±	**21.22***	17.67	±	3.678	19.89	±	2.92	4.157	$p < 0.05$
Time spent in intermediate area (s)	77.99	±	6.80	93.03	±	13.28	60.68	±	7.60	73.33	±	8.24	2.100	n.s.
Time spent in peripheral area (s)	196.68	±	9.94	**144.43**	±	**22.43***	228.93	±	8.48	206.83	±	10.14	5.571	$p < 0.01$

Results are expressed as mean ± S.E.M. of n = 10 mice/group. Bonferroni's *t*-test was used for multiple comparisons.
im, intra-muscular; n.s., not significant.
* $p < 0.05$, statistical significant difference from controls.

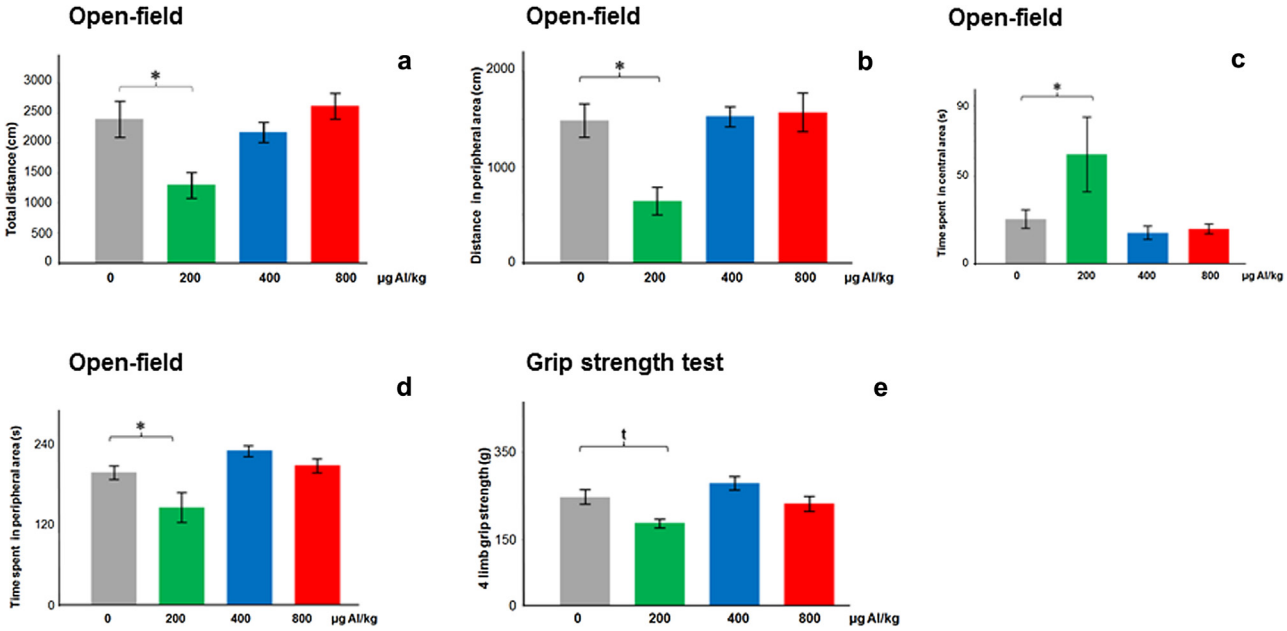

Fig. 1. Effects of different doses of Alhydrogel® on mouse behaviour. Altered scores were selectively observed with low Alhydrogel® doses. (a) Total distance in the open field; (b) distance in the peripheral area in the open field; (c) time spent in the central area in the open field; (d) time spent in the peripheral area in the open-field; (e) 4 limbs grip strength; 10 mice/group, results expressed as mean ± S.E.M, ANOVA test with post-hoc Bonferroni's test. * $p < 0.05$, statistical significant difference from controls; ^{t} $p < 0.10$, statistical tendency difference from controls.

tests (ANOVA or Student's *t*-test) can be used when normality distribution is assumed $p > 0.05$, whereas we used non-parametric test (Kruskal-Wallis test) when normality distribution is not assumed ($p < 0.05$). Data from behavioural tests were analyzed using a one-way analysis of variance (one-way ANOVA). Post hoc comparisons have been performed using the Bonferroni's test when Anova was significant. Data from microglia IHC were analyzed using a Student's *t*-test. Data from Al concentration measurement were analyzed using a non-parametric Kruskal-Wallis test followed by a Mann–Whitney procedure modified for multiple comparisons when appropriate. Significance was set at $p < 0.05$. All statistical analyses were carried out using SPSS 16.0 software (SPSS INC., Chicago, IL, USA).

3. Results

3.1. Body weight

The initial body weight was 30 g. Animals were weighed once a week during the whole procedure. No effects of treatment were observed on body weight (data not shown).

3.2. Behavioural tests

3.2.1. Open-field

In the open-field (Table 1), a one-way ANOVA showed a significant difference of the total distance walked ($p = 0.012$), the distance in peripheral area ($p = 0.002$), and time spent in both central ($p = 0.013$) and peripheral ($p = 0.003$) areas (Fig. 1a–d). Bonferroni's post hoc analysis showed that mice from the group Alhydrogel® 200 µg Al/kg crossed a significantly smaller total distance ($p = 0.026$) and distance in the peripheral area ($p = 0.005$) (1303.48 ± 213.04 cm and 643.61 ± 142.06 cm respectively) than controls (2401.01 ± 300.62 cm). Furthermore, animals injected with Alhydrogel® 200 µg Al/kg spent more time ($p = 0.047$) in the central (62.34 ± 21.22 s) and less ($p = 0.044$) in the peripheral areas (144.43 ± 22.43 s), as compared to controls (respectively 25.30 ± 5.17 s and 196.68 ± 9.94 s).

3.2.2. Elevated o-maze

In the elevated O-maze (Table S1 in the Supplemental data section) no significant differences between groups were observed across all measured variables.

3.2.3. Novel object recognition test

On the novel object recognition test (Table S2 in the Supplemental data section), one-way ANOVA did not reveal any statistical significant difference between groups across all studied variables.

3.2.4. Grip strength test

In the grip strength test (Table 2), significant difference ($p = 0.011$) between groups was observed for the 4-limb grip strength (Fig. 1e). Animals injected with Alhydrogel® at 200 µg Al/kg tended ($p = 0.076$) to have less strength (187.24 ± 9.84 g) compared to controls (246.76 ± 16.46 g).

Table 2
Effects of different doses of Alhydrogel® on muscular performances assessed in the grip strength test.

Grip strength test	Control			Alhydrogel® 200 µg/kg			Alhydrogel® 400 µg/kg			Alhydrogel® 800 µg/kg			ANOVA	
	mean	±	sem	mean	±	sem	mean	±	sem	mean	±	sem	$F_{(3,39)}$	*p*
Fore limbs (g)	171.69	±	6.36	162.85	±	10.68	157.33	±	7.14	160.20	±	6.61	0.788	n.s.
4 limbs (g)	246.76	±	16.46	**187.24**	±	**9.84**^{t}	278.59	±	15.95	231.97	±	17.32	4.188	$p < 0.05$

Results are expressed as mean ± S.E.M. of n = 10 mice/group. Bonferroni's *t*-test was used for multiple comparisons.
im, intra-muscular; n.s., not significant.
^{t} $p < 0.10$, statistical tendency from controls.

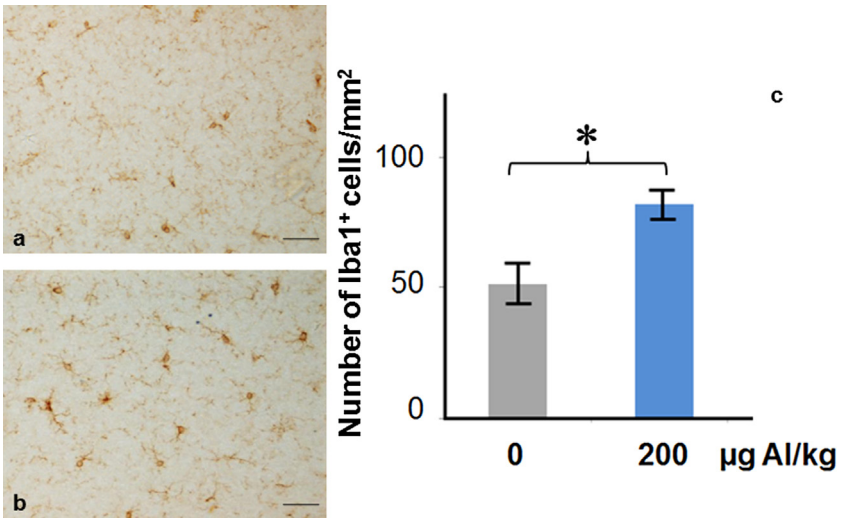

Fig. 2. Iba1$^+$ microglial cell density in the ventral forebrain. Iba-1 immunostaining showed a slight increase of the microglial cell density in the group of mice injected with Alhydrogel® 200 µg Al/kg; (a) control mice injected with PBS; (b) mice injected with Alhydrogel® 200 µg Al/kg; (c) quantification of the microglial cell density. 3 mice/group; results expressed as means ± S.E.M, ANOVA test with post-hoc Bonferroni's test * $p < 0.05$; scale bars: 50 µm.

Table 3
Aluminum cerebral concentration measured by furnace atomic absorption spectrometry (µg/g of dry weight).

Cerebral Al concentration	Control	Alhydrogel® 200 µg/kg	Alhydrogel® 400 µg/kg	Alhydrogel® 800 µg/kg	Kruskal-Wallis
	0.0200 *(0.0152–0.2088)*	**1.0027*** *(0.3368–1.1493)*	0.0143 *(0.0127–0.0200)*	0.0156 *(0.0137–0.3970)*	0.017

Results are expressed as median and quartiles (in brackets) of n = 5 brains/group. Non parametric Kruskal-Wallis test followed by a Mann-Whitney procedure was used for multiple comparisons.

3.2.5. Wire-mesh hang test, accelerating rotarod, hot plate test and tail suspension test

No statistical differences were observed between the 4 experimental groups for these 4 tests (Tables S3–S6 in the Supplemental data section).

3.3. Microglia immunohistochemistry

As shown in Fig. 2, Alhydrogel® injections at doses of 200 µg Al/kg induced a significant increase ($p = 0.033$) in the number of Iba-1$^+$ microglial cells in the ventral forebrain (81.90 ± 5.30 cells/mm^2) compared to controls (51.43 ± 7.87 cells/mm^2). Microglial density was similar to controls in visual and motor cortex and inferior colliculus in all groups. Microglial cell body size was similar in all groups (data not shown).

3.4. Cerebral Al level

The measurement of cerebral Al levels (Table 3) revealed a significantly ($p = 0.011$) higher Al level in brains from animals injected with 200 µg Al/kg (median value 1.00 µg/g of dry weight) than in brains from control group (0.02 µg/g of dry weight). No significant increase was observed in animals injected with 400 or 800 µg Al/kg (Fig. 3).

3.5. Muscle analysis

Granulomas with aluminium accumulations within macrophages were detected by Morin stain in the injected muscle of 6 animals (Fig. 4). As shown in Table 4, granulomas were found in 3/3 mice injected with 800 µg Al/kg, 3/3 mice injected with 400 µg Al/kg, and 0/3 mice injected with 200 µg Al/kg. The highest granuloma size was detected in mice injected with 800 µg Al/kg (Fig. 4). An unusual aspect reminiscent of aluminium adjuvant-induced pseudo-lymphoma (Maubec et al., 2005) was observed in one case of the 800 µg Al/kg group and in another one of the 400 µg Al/kg group. The lesion appeared as a dense central area filled with monocyte-like and small lymphocytic cells and a rim of large macrophages with clear cytoplasm (Fig. 4a), in which aluminium was accumulated (Fig. 4b, c). Medium-sized CD11b-expressing monocyte lineage cells were found throughout the dense area of the lesion (Fig. 4d, e) often mixed with abundant nuclei of other mononuclear cell types as assessed by DAPI staining (Fig. 4d). Multinucleated giant cells were not found.

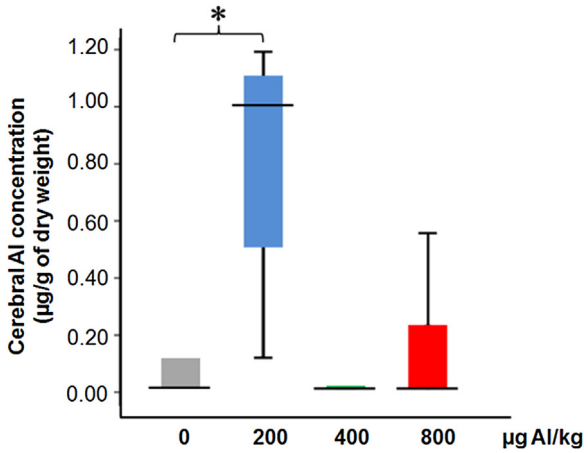

Fig. 3. Aluminium level determination in brain (µg/g of dry weight). Increased cerebral concentrations of aluminium were selectively observed with 200 µg/kg low Alhydrogel® dose. 5 mice/group; results expressed as median and range values, with quartiles boxes; non parametric Kruskal-Wallis test followed by Mann-Whitney test. * $p < 0.05$.

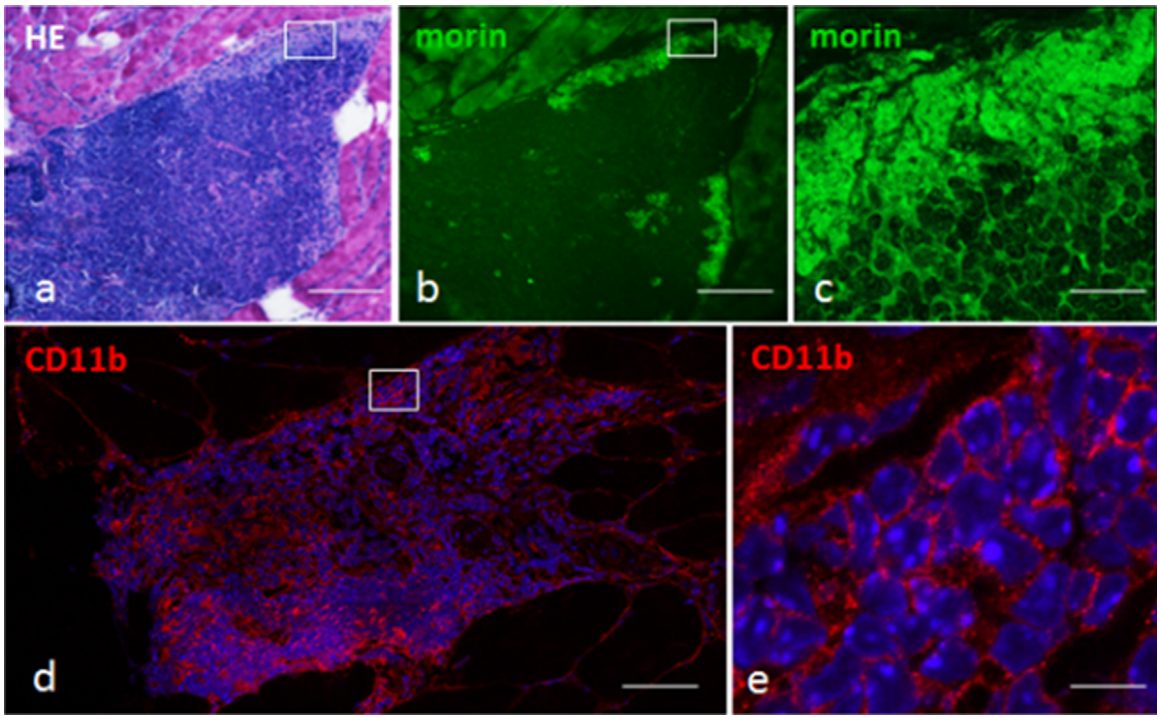

Fig. 4. Muscle sections 6 months after Alhydrogel® injections (800 μg Al/kg).
(a) Pseudolymphomatous lesion including a dense central area filled with mononuclear cells and a rim of macrophages with clear cytoplasm (HE: haematoxyline eosin, scale bar: 40 μm); (b,c) the rim of macrophages is selectively associated with aluminium accumulation stained in green (Morin stain, scale bars: 40 μm and 100 μm respectively); (d) CD11b-expressing monocyte lineage cells are present throughout the dense area of the pseudolymphomatous lesion, mixed with abundant DAPI⁺ nuclei of other mononuclear cell types (scale bar: 10 μm); (e) CD11b-expressing cells in an area prominently composed of medium-sized monocyte-lineage cells (scale bar: 20 μm).

Table 4
A semi-quantitative study of the granuloma size in injected muscle with Alhydrogel®.

Alhydrogel® group	No granuloma (0)	1 to 3 small granuloma (+)	>3 small granuloma (++)	>3 large granuloma (+++)
200 μg Al/kg	3	0	0	0
400 μg Al/kg	0	0	3	0
800 μg Al/kg	0	1	1	1

According to their size, observed granulomas were divided to four types: without granuloma (0), 1 to 3 small (+), >3 small (++) and >3 large (+++) granuloma. Then, number of animals of each criteria was determined, for n = 3 animals per group.

4. Discussion

In the present study, 8 widely used behavioural tests performed 180 days after im injections of 200, 400, or 800 μg Al/kg in form of Alhydrogel®, in adult female CD1 mice, showed significant effects restricted to animals exposed to the lowest dose. Animals injected with 200 μg Al/kg showed decreased locomotor activity levels assessed by lower total distance crossed in the open-field, as reported previously after subcutaneous injection of 100 and 300 μg Al/kg of Alhydrogel® (Petrik et al., 2007; Shaw and Petrik, 2009), with concomitant decrease of the grip strength test suggestive of moderate motor weakness. In addition, increase of time spent in central area concomitantly with a decrease of both walked distance and time spent in peripheral area pointed to a behavioural change impacting the protective aversion of rodents for open spaces (Bourin et al., 2007), whereas other studies have reported increased anxiety levels (Petrik et al., 2007; Agmon-Levin et al., 2014). In sharp contrast, the highest doses of 400 and 800 μg Al/kg did not cause such changes. Consistently with the altered behavioural tests, microglial cell density appeared significantly increased in animals exposed to 200 μg Al/kg. This mild cerebral innate immune activation was selectively observed in ventral forebrain including the amygdaloid nuclei, which are implicated in aversion/anxiety-like behaviours (LeDoux, 2007). Moreover, Al cerebral levels were significantly increased in animals injected with 200 μg Al/kg, but not in those injected with 400 and 800 μg Al/kg doses which showed neither neurobehavioural changes nor microglial reaction. The increased level of aluminium in brain was associated with an almost complete disappearance of aluminium-induced granuloma in mice injected with 200 μg Al/kg, while granulomas were constantly detected in the muscles injected with 400 or 800 μg Al/kg. In addition to conspicuous granuloma formation, 2/6 of these animals exhibited a pseudo-lymphomatous aspect suggesting an unusually strong local immune reaction to the foreign material.

In the present study we did not assess the concentration of Al in other tissues such as blood. Indeed, by using isotopic 26Al, it was previously shown that the maximal increase in the plasma Al within 28 days after Al hydroxide im injection in the rabbit was about 2 ng/mL. Since the normal Al concentration was about 30 ng/mL in the animal, it was said that such a small increase would have been masked by the Al background if 26Al-labelled adjuvants were not used (Flarend et al., 1997). Thus, Al plasma level determination on the long term, i.e. 6 months after im injection, cannot provide information in our mice. Furthermore in the present study the proposed method whereby Al is transported to organs and tissues

which are distant from the injection site does not actually involve the dissolution of the Al adjuvant into the muscle interstitial fluid and thereafter the blood but we are proposing that the transport of significant amounts of Al takes place in those cells which have infiltrated the injection site and taken up Al by endocytosis. Considering measurements of Al in muscle biopsies, we thought that they would not discriminate between extracellular and intracellular Al.

Evidence of a non-linear dose response curve of the neurotoxic effects of Alhydrogel®, with selective toxicity of the lowest dose used in the study challenges the classic toxicology paradigm "the dose makes the poison". Non-monotonic dose-response curves have been previously reported in the field of aluminium toxicology. Non-monotonic biphasic neurotoxic effects have been observed both in vitro (see for review Exley and Birchall, 1992; Platt et al., 1995; Eidi et al., 2015) and in vivo (Kumar, 1998; Tsunoda and Sharma, 1999; Kim, 2003; Roig et al., 2006) after oral Al administration. However, the dose-response curve of the present study was not biphasic. Moreover, since cerebral aluminium level was not increased in mice injected with 400 or 800 μg Al/kg, the lack of neurotoxicity observed with these high doses was likely due to limited Al cerebral translocation, rather than to its paradoxical cytotoxic effects on neural cells. This puzzling result is challenging in the absence of solid knowledge on Alhydrogel® pharmacokinetics. We previously studied the fate of aluminium particles following im injections. Aluminium hydroxide is a highly hydrated crystalline compound composed of elementary nano-needles of approximately 2.2 nm × 4.5 nm × 10 nm (Mao et al., 2013) and displays a fibrous morphology at transmission electron microscopy (Shirodkar et al., 1990; Eidi et al., 2015). This compound spontaneously forms micron-sized agglomerates (Johnston et al., 2002), subjected to slight size variations after antigen adsorption (Eidi et al., 2015) and in vivo interactions with phosphate, organic acid and proteinaceous environments. A series of recent reports from our laboratory have shown that translocation of aluminium hydroxide may be specifically related to monocyte lineage cell uptake of this poorly biodegradable compound (Khan et al., 2013; Crépeaux et al., 2015; Eidi et al., 2015), likely resulting from phagocytosis or macropinocytosis (Mao et al., 2013).

Recent studies suggest that the adjuvant effect requires uptake by dendritic cells (Morefield et al., 2005) and combines i) local up-regulation of chemokines, including CCL2 (MCP-1) and CCL3 (MIP-1α), that increase the recruitment of immune cells into the injection site; ii) increase of antigen uptake by innate immune cells; iii) induction of monocyte differentiation into dendritic cells, and iv) facilitation of migration of dendritic cells towards the dLNs to prime adaptive immune responses (Seubert et al., 2008). Macrophages capture bacteria which are usually in the 1–4 μm size range (Kowalski et al., 1999). A previous report showed in vitro exposure of monocyte lineage THP1 cells to Alhydrogel® 200 μg Al/mL resulted in cellular incorporation of Alhydrogel® agglomerates, the size of which was 1.20 μm as measured by transmission electron microscopy after 24 h (Mold et al., 2014, 2016). Consistently, this size range was shown to be optimal for particle uptake by mouse peritoneal macrophages (1–2 μm) (Tabata and Ikada, 1988) and for particle attachment and subsequent internalization by mouse alveolar macrophages (2–3 μm), whereas internalization markedly drops when the size exceeds 4.2 μm (Champion et al., 2008).

Alhydrogel® biopersistence was confirmed in a variety of laboratory animal models up to 6–12 months post-injection, in both the injected muscle (Verdier et al., 2005; Authier et al., 2006; Khan et al., 2013; Eidi et al., 2015) and distant lymphoid organs (Crépeaux et al., 2015). Particles traffic from an injected tissue to the dLNs is size-dependent, smaller particles (20–200 nm) being able to drain in a free form whereas medium-sized particles (0.5–2 μm) are exclusively subjected to cell transportation (Manolova et al., 2008). Although the point has not been precisely addressed in the literature for particles >2 μm, it seems possible that rapid cellular uptake of limited size particles is associated with quicker cell transportation to dLNs compared to large particles subjected to slow cell uptake, showing that, in this period of time, lower doses of adjuvant can diffuse in the body and reach the brain whereas higher ones do not, for a considered time point (Crépeaux et al., 2015).

On these grounds, we performed an exploratory evaluation of the size of agglomerates, a parameter that could be modified when concentration of the colloid suspension is increased to adjust doses (0.1, 0.2, 0.4 g Al/L in PBS 1X corresponding to 200, 400, and 800 μg Al/kg respectively). Dynamic light scattering showed that the colloid suspensions in PBS at pH 7.2 corresponding to the neurotoxic 200 μg Al/kg condition was exclusively composed of small bacteria-size agglomerates (mean = 1750 ± 100 nm), easily captured by innate immune cells. In contrast, suspensions corresponding to higher doses showed 2 size peaks, including one peak corresponding to very large agglomerates (about 35,000 nm) and another one corresponding to either small agglomerates (mean = 1500 ± 400 nm in the 400 μg Al/kg condition) or medium-sized agglomerates (mean = 4800 ± 500 nm in the 800 μg Al/kg condition).

Although further studies are clearly required to document the influence of Alhydrogel® agglomeration state on in vivo neurotoxic effects, such a finding would not be unprecedented in the field of particle toxicology since both cellular uptake and distribution in the body of other types of particles are influenced by the particle size (Buzea et al., 2007; Reddy et al., 2007; Landsiedel et al., 2012), and aggregation rate (Mühlfeld et al., 2008), two parameters that strongly determine particle toxicity (Bell et al., 2014; Leclerc et al., 2012; Nascarella and Calabrese, 2012; Mold et al., 2016).

In conclusion, the non-linear dose-response profile documented herein, in which the lowest dose but not the highest doses is neurotoxic in mice, is a novel insight in the field of aluminium adjuvant safety. It may suggest that Alhydrogel® toxicity obeys the specific rules of particle toxicology rather than any simplistic dose-response relationship. As a possible consequence, comparing vaccine adjuvant exposure to other non-relevant aluminium exposures, e.g. soluble aluminium and other routes of exposure, may not represent valid approaches. For example, aluminium retention rate observed after intravenous injections of traceable soluble aluminium citrate (Priest, 2004) has been used to set up the reassuring infant retention model of aluminium adjuvants (Mitkus et al., 2011). This model was based on the hypothesis that aluminium adjuvants are solubilized by citrate ions in muscle interstitial fluid (Flarend et al., 1997), without any consideration of quick adjuvant cellular uptake and systemic long term diffusion of adjuvant agglomerates (Khan et al., 2013; Eidi et al., 2015). In the context of massive development of vaccine-based strategies worldwide, the present study may suggest that aluminium adjuvant toxicokinetics and safety require reevaluation.

Competing interests

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2016.11.018.

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