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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.