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Analytical methodology

Aluminium (Al) speciation in serum and urine after subcutaneous venom immunotherapy with Al as adjuvant

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ABSTRACT

Background: Aluminium is associated with disorders and is the commonly used vaccine adjuvant. Understanding the mechanisms of how Al is transported, metabolized or of its toxicity depends on the knowledge of Al-interactions with bioligands, i.e. Al-species. Al-speciation in serum is difficult because of low concentration and the risk of exogenous Al contamination. Furthermore, Al-measurements may be hampered according to various interferences. This study aims for developing quality controlled protocols for reliable Al- and Al-species determination and for investigating probable differences in Al (-speciation) after Al-containing subcutaneous immunotherapy (SIT).

Methods: Sample donors were recruited either for the control group ("class-0", they never had been treated with SIT containing an Al-depot extract) or for the SIT-group ("class-1", they previously had been treated with SIT for insect venom allergy with an Al-depot extract). Blood was drawn for medical reasons and serum prepared. Additionally, some sample donors collected 24-h-urine. They had been informed (and they consented) about the scientific use of their samples. The study was approved by the ethic committee of the "Medical Association Westphalia-Lippe" and of the University of Münster, evaluating the study positively (No. 2013-667-f-S).

We applied quality controlled sample preparation and interference-free Al detection by ICP sectorfield-mass spectrometry. Al-species were analysed using size-exclusion-chromatography-ICP-qMS.

Findings: Al-concentrations or speciation in urine samples showed no differences between class-0 and class-1. Al-citrate was the main uric Al-species. In serum elevated Al-concentrations were found for both classes, with class-1 samples being significantly higher than class-0 ($p = 0.041$), but class-0 samples being approximately 10-fold too high compared to reference values from non-exposed persons. We identified gel-monovettes as contamination source. In contamination-free samples from HNO_3-prewashed gel-free monovettes ($n = 27$) there was no difference in the serum Al concentration between the two patient groups ($p = 0.669$)

Interpretation: Thorough cleaning of sample preparation ware and use of gel-free monovettes is decisive for an accurate Al analysis in serum. Without these steps, wrong analysis and wrong conclusions are likely. We conclude that gel-monovettes are unsuitable for blood sampling with subsequent Al-analysis. Whether Al in serum is elevated after SIT treatment containing an Al-depot extract, or not, remains inconclusive as the non-contaminated sample size was small.

1. Introduction

Repeated inquiries about a possible risk from aluminium in biomedical drugs had recently motivated the *Paul-Ehrlich-Institut* to provide the current state of knowledge on the safety of aluminium-containing adjuvants in extracts for allergen-specific immunotherapy [1]. Such actual inquiries are caused by reports where Al is associated with clinical disorders, e.g. in renal patients. Actually, high exposure of aluminium is recognized to be neurotoxic for more than a century and is discussed to be a toxic factor in several human diseases [2]. Its accumulation by patients with renal failure is a well-known hazard [3–5].

Despite similarities of Al-induced encephalopathy and Alzheimer's Disease (AD) in many symptoms, discussions about Al exposure and cognitive decline are still controversial. Specifically, because not all AD patients have elevated Al levels [6] while familial AD patients showed higher Al concentrations than all previous measurements of aluminium

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in brain [7], the discussion on Al being cause or consequence of AD is not decided. For complementing the data basis, reference [8] conducted speciation studies in serum to study the involvement of metals, including aluminium, in the progression of Alzheimer's disease. They found Al increased in demented patients but also evidence for a complex interdependency between different metals.

Notably, the aluminium load to humans is multi-factorial. Several routes for aluminium exposure to humans are known with Al from nutrition being generally accepted as the predominant source. The *European Food and Safety Agency* (EFSA) reports about 1.6–13 µg aluminium per day [9]. Another Al-source in discussion is the use of Al-containing anti-transpirants. Based on Al skin penetration rates from human studies [10] the German *Bundesintitut für Risikobewertung* (BfR) calculated an Al-uptake of 10.5 µg/d for a 60 kg person [11]. Additionally to the above every-day exposure, patients receiving Al-containing SIT can get up to 15 injections per year, each containing between 0.1–1.25 mg aluminium [12,13], which calculates as 4–51 µg Al on a daily basis. This amount is a relevant add-on-top to the general every-day-exposure.

Aluminium is commonly used in authorized vaccines and allergen preparations for SIT because it appears to boost or potentiate the immune response to the injected vaccine or allergen. However, despite about 90 years of widespread use of aluminium adjuvants, medical science's understanding about their mechanisms of action on a molecular level is still poor. There is also a concerning scarcity of data on toxicology and pharmacokinetics of these compounds.

Nowadays there is a growing acceptance of the fact that the understanding of the mechanisms of how Al is transported and excreted or the mechanism of aluminium toxicity is decisively dependent on the knowledge of the in-vivo Al interactions with bioligands at a molecular level [14]. This implies the determination of the Al speciation, i.e., the particular chemical forms and their concentrations at site in which the element is transported and deposited in the human body [14–17].

Speciation of Al in human serum is an extremely difficult task because the basal levels of this element in serum are typically below 3–5 µg/L and these low concentrations are even fractioned in speciation analysis [18]. Even worse, the risk of significant exogenous Al contamination to samples and used laboratory material is very high [14,17–19]. Consequently, inadvertent contamination during sampling, storage, sample preparation and analysis of serum or urine can introduce considerable uncertainty in the determination of aluminium [20,21]. During measurement of aluminium in biological materials, in particular in plasma or serum, the risk of contamination is the major factor. The sample must therefore be handled with as few as possible preparation steps. Analytical recommendations from validated Al determination methods provided by the German Research Foundation (DFG) recommend thorough rinsing of all disposable containers used for sample preparation with diluted nitric acid and Milli-Q water before use [20]. Aside from contamination, interfered Al determination, leading supposedly to wrong-elevated determinations, should be strictly prevented. To date, in clinical laboratories mostly graphite furnace atomic absorption spectrometry (GF-AAS) is applied for Al determination. Unfortunately, this method can suffer from serum Al concentrations being below methodical threshold value of detection in the necessary 1/5 or 1/10 dilutions for measurements [22], or from interferences by high chloride content in samples [20]. Inductively coupled plasma mass spectrometry (ICP-MS) thus is the more powerful method of choice. However, the $[^{11}B^{16}O]^+$ -cluster interferes the ^{27}Al mono-isotope and can cause wrong-elevated results [23] in boron-rich samples such as urine (up to 3000 µg boron/L urine of non-exposed, healthy sample donors) [24]. Therefore, high resolution ICP-sectorfield-mass spectrometry (ICP-sf-MS) was recommended for total Al determination [21]. With this instrument practically all interferences can be resolved from the ^{27}Al-signal [23]. It is further essential providing documented proof about adequate quality control and quality assurance measures during the study for which analytical results are reported [25,26].

Biological monitoring of human aluminium exposure has been conducted with determination of total Al in urine, which is thought to indicate recent exposure, and determination of total Al in plasma or serum, which is thought to better reflect the aluminium body burden and long-term exposure [25].

1.1. Study aims

According to the frequent use of aluminium in vaccination and SIT and the questions regarding its toxic effects we intended to monitor aluminium in urine and serum of controls (class-0) and patients who previously had received subcutaneous immune therapy with an insect venom extract containing Al as an adjuvant (class-1). The primary aims of the study had a special focus on developing quality controlled protocols, i.e. a pre-analytical protocol avoiding Al-contamination during sampling and sample preparation, an interference free, validated total Al and Al-species determination, and finally investigating whether differences in total Al or Al-speciation will be detected between class-0 and class-1 groups.

2. Experimental

2.1. Sample donors

Sample donors were selected from patients receiving SIT with insect venoms who visited the Clinic for Skin Diseases – General Dermatology and Venereology of the University Hospital in Münster for a control examination during two 2-months recruitment periods. We recruited patients for the control group ("class-0", n = 23), who never had been treated with SIT containing an Al-depot extract, and for the SIT-group ("class-1", n = 18) from patients, who previously had been treated with insect venom extracts containing Al as $Al(OH)_3$. Each injection contained 1.13 mg Al as adjuvant. Blood samples were sent blinded to the laboratory in Munich for Al-analysis and Al-speciation. Additionally, patients collected 24-h-urine. Patient's consent for the participation on this observational study was obtained. The study protocol was approved by the ethical commission of the "Medical Association Westphalia-Lippe" and of the University of Münster.

2.2. Samples and sample preparation

Urine (24 h urine) samples were collected into HNO_3-precleaned containers (Nalgene®, VWR, Ismaning, Germany). Blood samples were drawn using gel-monovettes for the first sampling and gel-free monovettes from Sarstedt (Nümbrecht, Germany) for later sampling. Serum was prepared subsequently.

All plastic containers for sample preparation or sample storage were pre-cleaned by incubation in HNO_3 (2%) for 1 h and subsequent three-times flushing with double-distilled water.

Serum and urine samples were stored at −70 °C and were blinded sent on dry-ice to the analytical laboratory in Munich. Before use, samples slowly thawed overnight at 4 °C and were diluted 1:10 in Milli-Q water directly before measurement. The entire sample preparation was performed under laminar flow benches (clean room condition).

2.3. Chemicals

Aluminium and rhodium single standards for ICP-MS were from Spex CertiPrep (Stanmore, UK). NH_4Ac, HAc and HNO_3 (65%) were purchased from Merck (Darmstadt, Germany). HNO_3 was purified by subboiling destillation. $Argon_{liqu}$ was purchased from Air-Liquide, Düsseldorf, Germany. An Argon vaporizer at the tank provided Ar gas for ICP-MS systems.