Original Research Article

Endotyping Cellular and Humoral Immunoreactivity againstAluminum in Allergic Patients: A Retrospective Study

ABSTRACT

Background: Several publications report that aluminum is responsible for hypersensitivity reactions. There is no standardized lab exam that can endotype (determine thepathophysiology responsible for the phenotype) hypersensitivity to aluminum.
Aim: To evaluate the potential of the Tube Titration of Precipitins (TTP) and the Leukocyte Adherence Inhibition Test (LAIT) to endotype humoral and cellular immunoreactivity against aluminum in patients clinically diagnosed with allergic contact dermatitis, intrinsic atopic dermatitis, and/or non–IgE-mediated urticaria.
Study Design: We retrospectively examined the medical charts of two cohorts of 100 patients each; range 6 to 87 years; diagnosed with allergic phenotipes, who were investigated with the help of TTP (first cohort) or *ex vivo* challenge tests monitored by LAIT (second cohort) against aluminum.
Methodology: The registered results of TTP against 1 mg/mL aluminum solution were distributed in ranges through a cascade distribution chart to outline the variability of the results inside the first cohort. The registered results of the Leukocyte Adherence Inhibition (LAI) percentage promoted by the *ex vivo* challenges with 1 mg/mL aluminum solution were distributed in ranges through a cascade distribution chart to outline the variability of results inside the second cohort.
Results: Most TTP results concentrated on higher dilutions. The mean was estimated at 1:301; the median was 1:256; the standard deviation was estimated at 1:204; the mode was 1:512 (appeared 46 times).The LAI ranged from 0% to 88%. The mean was 52%; the median was 49%; the standard deviation was 23.9%; the mode was 60% (appeared five times). The cascade graphs demonstrate a wide range of distribution of TTP and LAI results.
Conclusion: Our preliminary results support that the TTP and LAIT performed with 1 mg/mL aluminum solution may discriminate diverse humoral and cellular immunoreactivity degrees in patients suffering from allergic contact dermatitis, intrinsic atopic dermatitis, and/or non–IgE-mediated urticaria.

Keywords: Aluminum; Endotype; Hypersensitivity; Leukocyte Adherence Inhibition; Leukocyte Adherence Inhibition Test; Phenotype; Precipitins; Precision Medicine.

Abbreviations:
LAI: Leukocyte Adherence Inhibition
LAIT: Leukocyte Adherence Inhibition Test
TTP: Tube Titration of Precipitins

1. INTRODUCTION

Aluminum (or aluminium) is a post-transition metal with the symbol Al, atomic number 13, and a relative atomic mass of 26.98, which has a great affinity to oxygen [1]. The American Contact Dermatitis Society elected aluminum as the "Allergen of the Year 2022"[2]. Aluminum may be present naturally in jewelry, piercings, cosmetics, tubes of toothpaste, medicines, tattoo inks, foods (at shallow levels), or due to using aluminum cooking utensils and food additives [3].

Some foods, such as potatoes,spinach, and some teas, may contain naturally higher levels of aluminum [4]. Remarkably, the mean concentration of aluminum in soy-based infant formulas is remarkably higher than in milk-based and corn-based infant formulas [5].The sixty-seventh meeting of the Joint FAO/WHO Expert Committee on Food Additives held in Rome in 2006 established the provisional tolerable weekly aluminum intake as 1 mg/kg body weight [6]. Aluminum in its metal presentation (E 173) and aluminum salts are bactericides frequently used as preservatives for foods and cosmetics [7]. Aluminum sulfates (E 520 to E 523) are mainly used in the food industry as thickeners and emulsifiers [8]. Aluminum-containing additives are also used for specific purposes: A) as raising agent inbaking powder, such asaluminum sodium sulfate (E 521) and sodium aluminum phosphate-acidic (E 541); B) as firming agents during the processing of jellyfish and pickles such as aluminum potassium sulfate (E 522); C) as emulsifiers in processed cheese, such as sodiumaluminum phosphate-basic (E 541); D) as anti-caking agents in powder mixesfor beverage mixers and non-dairy creamssuch as sodium aluminosilicate (E 554); E) as anti-caking agents for table salt and vanilla powder such as calcium aluminum silicate (E 556); F) as carriers for pigments (titanium dioxide and iron oxides)such as the potassium aluminum silicate (E 555) and aluminum powder(E 173) in decorating sugar-coated flourconfectionery candy coatings, also known as lakes [9-14].

Aseptic aluminum-containing plastic packages and cartons prevent oxidation and light damage to perishable food [15]. Elemental aluminum and its salts (such as sulfate, phosphate, hydroxide, and silicate) have a wide variety of uses, such as food additives, cooking utensils, foodpackaging (beverage cans and foil), water treatment, cosmetics (sunscreens), toiletry products (antiperspirants, tubes of toothpaste and sun blockers), antiacidswith aluminum hydroxide, anti-diarrheic with kaolin (hydrated aluminum silicate or E 559),producing contact dermatitis, granuloma, and urticaria in sensitized people [16-19]. Pool water flocculants are made with aluminum salts and may produce occupational allergies[20]. Aluminum-containing compounds have been employed as adjuvants in vaccines to amplify antigen-specific Th2 responses [21]. There is a positive dose-response relationship between plasmatic aluminum concentrations and cognitive impairment among workers with occupational exposure to aluminum [22]. Aluminum nanoparticles are associated with memory impairment and hippocampal inflammation induced by microglial activation,following upregulation of the inflammatory cytokine IL-1β (interleukin-1β)in mice[23]. Current lab models to study Alzheimer's disease are established through intraperitoneal injection of aluminum chloride ($AlCl_{3)}$in rats or aluminum nanoparticles in mice [24, 25]. Diagnosis of delayed hypersensitivity to aluminum is usually clinically made in patients with contact dermatitis with the help of cutaneous path tests performed with aluminum salts dispersed in petrolatum [26]. However, systemic aluminum immunoreactivity has already been documented by systemic immunoassay markers, such as eosinophilia [27].

The clinical suspicion of immediate and delayed hypersensitivity against aluminum is usually confirmed through skin allergy tests and patch tests, respectively. Until now, no widespread lab exam has been able to document specific immunoreactivity against aluminum to help with clinical diagnosis.

The Leukocyte Adherence Inhibition Test (LAIT) and the Tube Titration of Precipitins (TTP) are performed in our facilities as triage tests for suspected allergens prescribed before the performance of more exhaustive *in vivo* provocation tests [28-34].

The present study hypothesizes that the LAIT and the TTP may differentiate endotypes and degrees of immunoreactivity against aluminum among patients suffering from common allergic phenotypes. To evaluate the potential of the LAIT and the TTP to discriminatehumoral and cellular immunoreactivity against aluminum, we retrospectively compiled the electronic medical charts of patients clinically diagnosed with allergic contact dermatitis, intrinsic atopic dermatitis, and/or non–IgE-mediated urticaria who were investigated with these procedures.

2. MATERIALS AND METHODS

2.1 Subjects

After receiving Institutional Review Board approval from the Instituto Alergoimuno de Americana (Brazil; 06/2024), we proceeded with the electronic chart review of 9,200 outpatients who attended our facility from January 2018 to August 2024.

The first cohort of 100 outside patients had been submitted to TTP with 1 mg/mL of aluminum solution for presenting allergic contact dermatitis, intrinsic atopic dermatitis, and/or non–IgE-mediated urticaria. This cohort counted 24 males and 76 females; mean age 38.4 years; SD 20.4 years; range 6 to 87 years; median 36.5 years; mode = 46 (appeared five times); geometric mean = 32 years.

The second cohort of 100 outside patients had been submitted to an *ex vivo* allergen challenge test with aluminum solution 1mg/mL monitored with LAIT for presenting allergic contact dermatitis, intrinsic atopic dermatitis, and/or non–IgE-mediated urticaria. This cohort counted 28 males and 72 females;mean age 40.3 years; SD 17.5 years; range 8 to 75 years; median 42 years; mode = 42 (appeared six times); geometric mean = 35.2 years.

This study did not include patients under biological and/or systemic anti-inflammatory therapy. These procedures were offered to patients with clinical suspicion of aluminum hypersensitivity who demonstrated a non-reactive or inconclusive skin test against aluminum 1 mg/mL solution [35].The cascade graphs were mounted using the functionalities of the spreadsheet editor Microsoft Excell® software.

2.2 Aluminum solution

The aluminum solution was prepared with powdered acetate aluminum diluted with distilled water at 1 mg/mL to perform the allergic skin tests, TTP, and LAIT.

2.3 *Ex vivo* Investigation: Leukocyte Adherence Inhibition Test

2.3.1 Procedure for allergen *ex vivo* challenging

We performed the LAIT as previously described [29, 30, 36-45]. Shortly, each donor's fresh plasma was divided into two parts and used in paralleled *ex vivo* challenging tests with aluminum acetate solution 1 mg/mL and the unchallenged plasma assay. We collected the plasma with high leukocyte content (buffy coat) from the heparinized tube after one hour of sedimentation at 37 °C. Then, we distributed aliquots of 100 µL into Eppendorf tubes kept under agitation for 30 minutes (200 rpm at 37 °C) with aluminum solution (10µL of a solution with 1mg/mL) or without aluminum solution (when used as control).

2.3.2 Procedure for adherence assay

After incubation, the plasma was allocated into a standard Neubauer hemocytometer counting chamber with a plain, non-metallic glass surface and left to stand for 2 hours at 37 °C in the humidified atmosphere of the covered water bath to allow leukocytes to adhere to the glass. Next, we counted the leukocytes, removed the coverslip, and washed the chamber by immersion in a beaker with PBS at 37 °C. Then, we added a drop of PBS to the hemocytometer's chamber and allocated a clean coverslip over it. The remaining cells were counted in the same squares as previously examined.

2.3.3 Procedure for calculation

The percentage of Leukocyte Adherence (LA) of each assay was estimated as: (the number of leukocytes observed on the hemocytometry chamber after washing divided by the number of leukocytes observed on the hemocytometry chamber before washing) and multiplied by 100 (%). The Leukocyte Adherence Ratio (LAR) was estimated based on the ratio between the LA from the antigen-specific challenged plasma and the LA from the unchallenged control plasma: LAR = LA of the challenged sample divided by LA of unchallenged control plasma multiplied by 100 (%). To further calculate the Leukocyte Adherence Inhibition (LAI), we subtracted the LAR from 100 (%). We employed the LAI results for the cascade distribution chart and the statistics calculations, both performed with the help of the Microsoft Excel® statistical package.

2.4 *In vitro* Investigation: Tube Titration of Precipitins (TTP)

As previously reported, the semi-quantitative TTP against the aluminum solution was performed in a transparent vitreous tube [46]. Shortly, the patient's blood was collected in a clot-activator collecting tube. After separation, the serum was centrifugated at 2,000 rpm for 10 minutes. The allergen extracts were allocated in sets of eleven glass tubes at progressive duplicated serum dilutions. The progressive dilutions were combined with the 15 µL of the

antigen (1 mg/mL) with 250 µL of the patient's serum, progressively diluted into physiological saline solution (NaCl 0,9%) in the dilution ratios of 1:1; 1:2; 1:4; 1:8; 1:16; 1:32; 1:64; 1:128; 1:256; and 1:512. One tube was a blank control done with the water and serum to observe occasional spontaneous precipitation (Sia Test). After 24 hours, the tubes were examined, and the titers (the highest dilution factor that yields a positive reading) were recorded [47].

3. RESULTS

As a retrospective survey, there was no research protocol; therefore, we report the incidental immune investigation as registered in the digital medical charts.

The cascade distribution graph showed a wide distribution range of TTP results. There was one negative result. Most positive results concentrated on the higher dilutions (Fig 1). The mean was estimated at 1:301; the median was 1:256; the standard deviation was estimated at 1:204; the mode was 1:512 (appeared 46 times). All Sia tests were negative.

The LAI ranged from 0% to 88%. The mean was 52%; the median was 49%; the standard deviation was 23.9%; the mode was 60% (appeared five times). The cascade distribution graph demonstrates a wide range of distribution of LAI results (Fig.2). Three patients ignored the presence of the allergen on the plasma and presented no inhibition of leukocyte adherence (LAI = 0%) after contact with the aluminum solution. Some patients showed low or moderate immunoreactivity during the *ex vivo* challenge test, while most displayed strong immunoreactivity, suggesting aluminum's participation in the hypersensitivity condition.

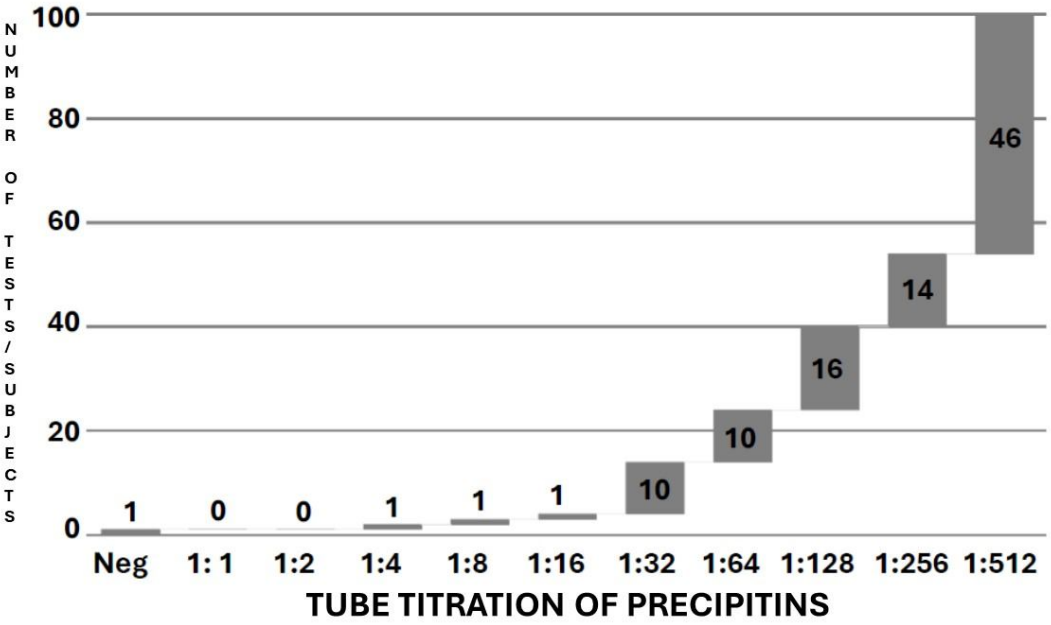

Fig. 1. Cascade distribution chart of the Tube Titration of Precipitins (TTP on the x-axis) resulting from the aluminum solution against the serum of a cohort of 100 tests/subjects (y-axis).

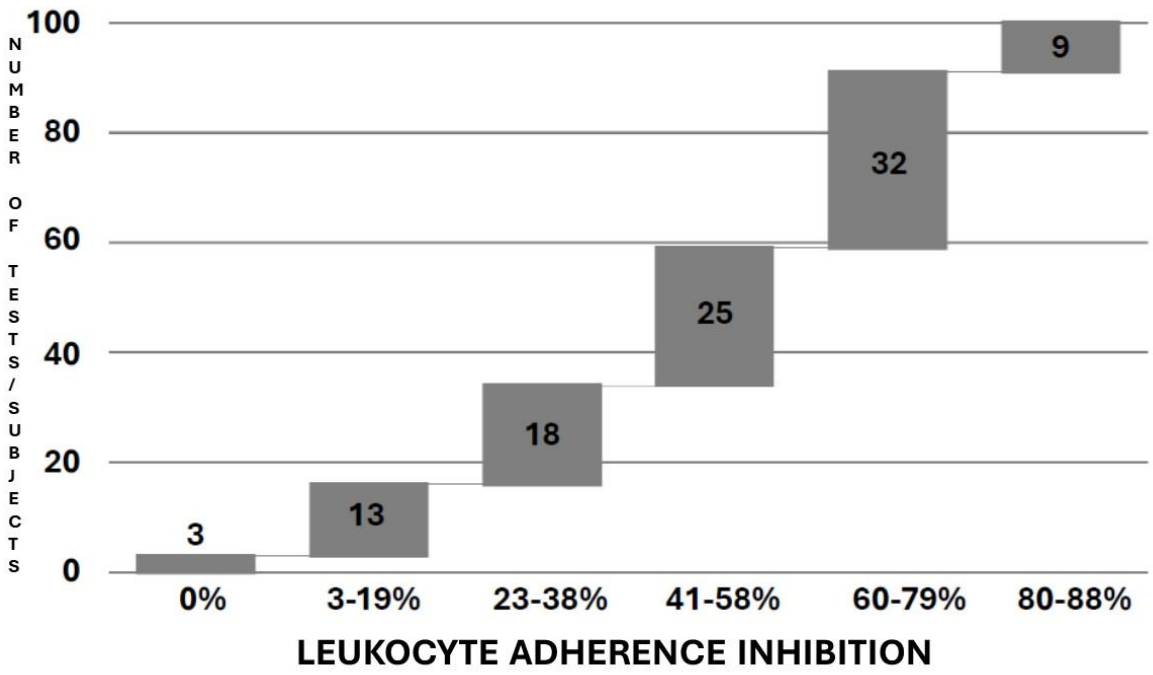

Fig. 2. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (LAI) results (x-axis %) of *ex vivo* aluminum solution monitored by the Leukocyte Adherence Inhibition Test (LAIT), according to the respective number of outcomes over a cohort with 100 tests/subjects (y-axis).

4. DISCUSSION

Aluminum was one of the first inorganic adjuvants envisaged to boost the immune response of infectious vaccines and subcutaneous allergen immunotherapies [48].The first description of using aluminum salts to boost immune response was published in 1924 when used as an adjuvant to tetanus and diphtheria toxoids [49].Further (1938), aluminum salts were presumed as adjuvants to subcutaneous allergen immunotherapies fortheir depoteffect, increasing the permanence of the immunotherapeutic agent on the site of the injection, allowing more time for the innate immune system to process the antigens (adsorptive adjuvant)[50]. Later (1985), it was established that the presence of aluminum salts increased the uptake of allergens by antigen-presenting cells, stimulating antigen-induced T-cell proliferation [51]. Aluminum-containing adjuvants also activate innate immune response by stimulating dendritic cells, inducing $CD4^+$ T celldifferentiation [52].Aluminum acts as a vaccine adjuvant by producing cellular necrosis thatreleases inflammatory cytokines (such as IL-33), stimulating innate and adaptive immunity [53]. It is a corollary to think that ingestion, contact, and parenteral administration of this metal may produce residual asymptomatic or even symptomatic conditions in a society "drowned" in aluminum through food additives, cosmetic preservatives, medicines, vaccine adjuvants, and others [54]. Aluminum hypersensitivity mightrepresent just the iceberg's tip of an organism saturated by its effects, alerting the patient's conscious mind to avoid contact and ingestion.

Personalized medicine is an approach dedicated to diagnosing the endotypes responsible for disease phenotypes. [55]. While phenotypes are defined by the visible clinical manifestations of the conditions(some of these induced by multiple underlying mechanisms), an endotype is a subtype of a given phenotype defined by its pathophysiological mechanism[56]. The classical four hypersensitivity mechanisms described by Gell and Coombs in the sixties are now amplified to seven types with

several subtypes, implying an increased responsibility of the clinical caretakers to investigate the mechanisms responsible for the disease phenotypes in order to recommend better strategies to avoid the allergens and to prescribe tailored treatments for these infirmities [57, 58]. Endotyping the hypersensitivity mechanisms may also help distinguish superimposable phenotypes presenting similar symptoms that may hamper establishing a precise diagnosis [59]. To detect immune responses against aluminum, we spreadsheeta retrospective compilation of data produced at our facilities by TTP and TIAL, exploring humoral and cellular immunoreactivities against aluminum. TTP and LAIT are complementary triage tests used at our facilities to select worthwhile antigens to proceed with more laborious *in vivo* provocation tests when the specific IgE is undetectable and skin tests are inconclusive or unfeasible due to the patient's skin conditions.

These assays do not identify the exact immune mechanisms responsible for the clinical condition. Instead, they provide clues about sensitization and immunoreactivity distributed into an extensive spectral range between immune tolerance and symptomatic hypersensitivity. TTP and LAIT must be interpreted as triage immune markers of the humoral and cellular responses after contact with a specific antigen, configuring themselves as techniques to quantify an exposome measurement, as proposed by the exposome-wide association study [60].

This preliminary retrospective survey demonstrated extensive results from the TTP and the *ex vivo* challenge test monitored by LAIT against aluminum in two cohorts of patients with various allergic symptoms. None of our patients presented an exclusive reaction to aluminum. Every patient was simultaneously tested with several chemical and biological allergens, demonstrating positive results for some of them. Our results suggest that reactive allergic patients may impair their symptoms by an additional immunoreactivity against environmental or ingested aluminum.

5. LIMITATIONS

This study is a retrospective analysis of data collected over six years. There was no protocol research, and the subject's data were limited to the essentials available on our electronic sheets. Therefore, we could not establish a cross-comparison between positive and negative controls to validate the results. The number of subjects is appropriate for a preliminary study; however, future studies must be more comprehensive. The lack of a research protocol implies the possibility of a bias produced by the physician's point of view who indicated the exam (CEO) based on a clinical suspicion led purely by the anamnesis, physical examination, routine lab exams, and allergic skin tests. The study lost many of these patients to follow-up, so assuring the relationship between the immunoassays' results and the patient's clinical outcome is impossible.

6. CONCLUSION

Our preliminary results show that the TTP and LAIT may differentiate diverse degrees of immunoreactivity against aluminum in patients clinically diagnosed with non–IgE-mediated cutaneous allergies. This methodology can provide a socioeconomic impact since the methodologies to perform TTP and LAITare inexpensive and can be performed in a single lab attached to the facilities with minimum laboratory equipment. However, the propaedeutic meaning of these results and the possibility of interferents must be better established [61]. More studies focused on the quality-by-design approach with prospective larger double-blind cohorts need to evaluate the potential contribution of TTP and LAIT for endotyping immunoreactivity of patients suspected of symptomatic hypersensitivity against aluminum and other similar food processing additives [62].

ETHICAL APPROVALS

The authors have collected and preserved written ethical approval per international standards.

Disclaimer (artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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