Original Research Article

Contribution of the Leukocyte Adherence Inhibition Test to the Diagnosis of Immunoreactivity against Cobalt.

ABSTRACT

Aims: To evaluate the potential of the Leukocyte Adherence Inhibition Test (LAIT) as a tool for evaluating immunoreactivity against cobalt in patients with clinical suspicion of cobalt hypersensitivity.
Study Design: We retrospectively examined the medical charts of a population of 97 patients diagnosed with Allergic Contact Dermatitis (ACD) and/or dyshidrotic eczema (DE) with clinical suspicion of cobalt hypersensitivity who were investigated with an *ex vivo* challenge monitored by LAIT against $CoCl_2 \cdot 6H_2O$.
Place and Duration of Study: Instituto Alergoimuno de Americana – São Paulo – Brazil – between January 2018 and June 2023.
Methodology The percentage of Leukocyte Adherence Inhibition (LAI) promoted by the *ex vivo* challenges with $CoCl_2 \cdot 6H_2O$ was distributed in ranges through Cascade Distribution Charts to observe the variability of the results.
Results: The LAI mean was 44.8%, SD 26.9%, range 0% to 94%, mode = 0% (appeared 14 times). There was a wide range of distribution of LAI results, suggesting that some patients had immunoreactivity against cobalt, while others do not.
Conclusion: Our preliminary results support the fact that the LAIT performed with $CoCl_2 \cdot 6H_2O$ may differentiate diverse degrees of *ex vivo* immunoreactivity against cobalt in allergic patients.

Keywords: Cobalt, Dermatitis, allergic contact, Dermatitis, contact, Diagnosis, Eczema, dyshidrotic, *Leukocyte Adherence Inhibition Test, Skin tests.*

In abstract section should be one unity study design, duration study in methodology section

1. INTRODUCTION

Cobalt is a weakly reducing chemical element with the symbol Co, atomic number 27, and atomic weight of the stable isotope 58.9 Da, classified in the periodic table as a transition metal [1]. Cobalt is a highly reactive element with several oxidative states which can effectively compete with other cationic metals. It does not accumulate in the organism since it is rapidly excreted in the urine [2]. However, persistent exposure to high levels of cobalt may lead to an intoxication condition known as Systemic Cobaltism [3]. A cobalt-rich diet is also reported to play a role in patients with Dyshidrotic Eczema (DE) [4]. Cobalt is also a recognized cause of Allergic Contact Dermatitis (ACD) [5]. ACD is a T-cell–dependent skin disease manifested as a delayed-type hypersensitivity in which the allergen is introduced through intact skin [6]. The T helper lymphocytes recognize antigens via their specific T-cell receptors when presented by Antigen-Presenting Cells. After exposure to interleukin-12 naïve T helper lymphocytes evolve into T helper type 1 cells responsible for activation of the cell-mediated immunity. If directed to a chemical such as cobalt in the skin of a cobalt-allergic patient, a delayed type of hypersensitivity reaction develops and may manifest as ACD [7]. Cobalt may be absorbed

through the skin and gastrointestinal and pulmonary tracts (cobalt-containing dust in the industry). Inhalation of cobalt salts may cause rhinitis, and asthma [8].

There is no evidence that humans require inorganic cobalt [9]. Cobalt is essential to mammals only when constituting the active center of one of the organometallic cobalamin's forms, also described as the Vitamin B12 (VB12) vitamers [10]. The cobalamin consists of a corrinoid ring attached to a cobalt atom, which is, in turn, attached to a radical that defines its final configuration [11]. Methylcobalamin (also called Mecobalamin) and Adenosylcobalamin (also called Coenzyme B12) are both VB12 vitamers naturally found in food sources and are cofactors of the methionine synthase, and methyl malonyl-CoA mutase, respectively [12]. Mammals are not able to synthesize VB12, which is produced by fermentative bacteria in the digestive tract of ruminants, by homemade (or industrial) fermentation of edible vegetables, or by bioengineering in microbial chemical factories [13, 14]. So, humans obtain VB12 from animal-derived foods, fermented food, or industrialized supplements [15-17]. Hydroxocobalamin (also called Hydroxycobalamin) is extracted from microorganisms in laboratories to be administered via parenteral routes [18]. Cyanocobalamin is the synthetic VB12 vitamer that contains a cyanide molecule linked to the molecule to increase stability [19]. It is usually administered through oral supplements or fortified foods. Human lysosomes can convert these two last forms of VB12 into Methylcobalamin and Adenosylcobalamin to become metabolically active [20]. There is no report of hypersensitivity reactions to these two naturally occurring VB12 vitamers, however, synthetic cobalamin supplementation may be responsible for systemic hyperpigmentation dermatitis [21]. Some patients may be hypersensitive to cyanocobalamin, but not to hydroxocobalamin [22]. Others may develop an allergy to hydroxocobalamin, but not to cyanocobalamin [23]. These molecules are water-soluble, and the occasional excess is just eliminated by the urine or by the feces. However, it is not clear if, after absorption, the cobalamin liberates inorganic cobalt into human tissues. There is not (yet) any physiologic pathway explaining a link between cobalamin allergy and immune hypersensitivity to inorganic cobalt. These conditions present very distinct clinical pictures and the patients with both cobalt hypersensitivity and cobalamin hypersensitivity might have developed two distinct immune hypersensitivity mechanisms against two related allergens [24]. For the time being, this is just speculation deserving further attention [25]. However, cobalamin may suffer spontaneous degradation under industrial processing and storage, releasing free cobalt that may be present in cobalamin supplements [26]. Another possibility is the degradation of the corrinoid ring under the attack of digestive enzymes. After being released from animal-derived food, the cobalamins link to the haptocorrin, a pepsin-resistant glycoprotein produced in saliva, that carries the cobalamin until the duodenum, where it links to the intrinsic factor to be absorbed. There is the possibility that an occasional overdose of cobalamin may supplant the haptocorrin availability and the unprotected cobalamin could be degraded liberating inorganic cobalt into gastric juice. Again, for now, just speculation justified by the fact that the haptocorrin deficiency courses with low serum cobalamin concentrations [27].

Inorganic metals such as cobalt, chromium, and nickel are strong sensitizers [28]. Metal allergies such as nickel hypersensitivity and cobalt hypersensitivity are usually associated and have a high prevalence in the general population, especially in dermatitis patients due to skin sensitization after prolonged contact [29]. People may be exposed to inorganic cobalt by dietary sources, cosmetic products, jewelry, cloth accessories, occupational hazards, environmental pollution, and medical procedures. The main dietary sources of inorganic cobalt are nuts (mainly Brazil nuts), yeast products, coffee, cocoa, and grains [30, 31]. Vegetables cultivated with wastewater may also represent a risk for cobalt exposure [32].

Cosmetics such as enamels, dark eyeshadows, face paints, hair creams, and henna dyes may be more or less rich in cobalt content, mainly when elaborated in countries lacking regulatory legislation [33]. Several cobalt salts are prohibited for cosmetic products in countries where the consumer's health is taken into consideration by responsible legislators [34]. Cobalt is incorporated in the forging of alloys to produce cloths accessories and jewelry such as wedding rings, necklaces, imitation jewelry (bijou), buckles, buttons, hooks, pins, rivets, snaps, zippers, earrings, and piercings, which release their metallic ions under sweating conditions [35]. Metal tools such as tweezers, sectioning clips, hair clips, and straight razors may also release nickel and cobalt producing DE and ACD, mainly in hairdressers [36].

Cobalt may be also found in pigments, stains, paints, and tattoo inks [37]. These pigments are used to paint ceramic and jewelry. Cobalt-containing blue ink was already reported as a cause of tattoo-associated widespread urticaria-like itching lesions histologically characterized by dermis edema and perivascular infiltration of lymphocytes, histiocytes, and eosinophil granulocytes [38]. Several pigments are elaborated from cobalt, each classified with a unique Colour Index (C. I.) generic

name and number. The most used is the Pigment Blue 28 (C. I. 77346) also known as Cobalt Blue ($CoAl_2O_4$). There is also the Pigment Blue 36 (C. I. 77343) also known as Cobalt Chromite Blue [$Co(Al, Cr)_2O_4$]. Cobalt participates in the Pigment Violet 14 (C. I. 77360) also known as Cobalt Violet Phosphate [$CO_3(PO_4)_2$]. Some green pigments are also elaborated with cobalt, such as the Pigment Green 19 (C. I. 77335) also known as Cobalt Nickel Zinc Titanite Green ($Co, Ni, Zn)_2TiO_4$, the Pigment Green 26 (C. I. 77344) also known as Cobalt Chromite Green ($CoCr_2O_4$) and the Pigment Green 50 (C. I. 77377) also known as Cobalt Titanate Green (Co_2TiO_4) [39].

The research on hypersensitivity to cobalt by patch test is mandatory in occupational contact dermatitis [40]. Cobalt is a very common causative agent of ACD in print machine workers (positive in 20.8% of patch tests) [41]. Construction workers may be exposed to cobalt, and similar sensitizing agents such as chromium and nickel, through cement, concrete, and mortar. The most common body part affected in these patients is the hand [42]. Workers from the hard metal industry (mining, forging, and grinding) are highly exposed to cobalt [43]. Production workers exposed to cobalt commonly develop ACD and/or DE in exposed body areas [44].

Cobalt-based alloys are usually elected to manufacture prosthetic biomaterials because they have better resistance to corrosion than stainless steel, greater resistance to fatigue, and a perfect balance between biocompatibility and mechanical properties [45]. The nonmagnetic molybdenum-cobalt–chromium alloy is, particularly, a common choice for knee implants, metal-to-metal hip joints, femoral components, and dental prosthetics, due to its excellent wear and corrosion resistance, strength, and biocompatibility [46]. Metal hypersensitivity is a great challenge to substitutive orthopedic surgery. The metal wear debris causes chronic inflammation, produces osteolysis, osteoclast production, and aseptic loosening, contributing to the development of Aseptic Lymphocyte-Dominated Vasculitis-associated Lesion, a type IV metal hypersensitivity response due to releasing of cobalt II metal ions which link as haptens to the proteins of synovial fluid [47].

2. MATERIALS AND METHODS

2.1 Subjects

After receiving Institutional Review Board approval, from the Instituto Alergoimuno de Americana (Brazil), we proceed with the electronic chart review of a population of 7,300 allergic patients who attended our outpatient facility from January 2018 to June 2023. A group of 97 patients had been submitted to an *ex vivo* allergen challenge test with cobalt monitored with LAIT. This procedure was offered to patients with extensive dermatitis preventing the accomplishment of the cutaneous tests or with a clinical suspicion of cobalt hypersensitivity associated with a non-reactive patch test. This was a very diversified cohort with 31 males, mean age 46.6 years, SD 20.4 years, range 18 to 85 years, mode = 42 years (appeared 6 times), geometric mean = 42.9 years.

2.2 Antigen preparation

The $CoCl_2 \cdot 6H_2O$ was acquired from Labcenter Campinas. The powder was weighed and diluted in a buffer solution [NaCl 10g, KH_2PO_4 0,72g, Na_3PO_4 2,86g, H_2O 600mL], to achieve the final concentration of 1 mg/mL to be employed in the LAIT.

2.3 *Ex vivo* Investigation: Leukocyte Adherence Inhibition Test

All patients were submitted to the *ex vivo* challenge tests monitored by the Leukocyte Adherence Inhibition Test (LAIT), against $CoCl_2 \cdot 6H_2O$ to evaluate the cellular response. The LAIT was performed as previously described [48-57]. Shortly, each donor's fresh plasma was divided into two parts and used in paralleled *ex vivo* challenging tests with $CoCl_2 \cdot 6H_2O$ and the unchallenged plasma assay. The plasma with high leukocyte content (buffy coat) was collected from the heparinized tube after one hour of sedimentation at 37 °C and aliquots of 100 µL were distributed into Eppendorf tubes kept under agitation for 30 minutes (200 rpm at 37 °C) with (or without, as used as control) antigen extract (10µL of a solution with 1mg/mL and pH 7.5). 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, leukocytes were counted, the coverslip was removed, and the chamber was washed by immersion in a beaker with PBS at 37 °C. A drop of PBS was added

to the hemocytometer's chamber and a clean coverslip was placed over it. The remaining cells were counted in the same squares as previously examined. 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 groups and the LA from the unchallenged control group: LAR = LA of the challenged sample divided by LA of the unchallenged control sample, multiplied by 100 (%). To further calculate the Leukocyte Adherence Inhibition (LAI) the LAR was subtracted from 100 (%). The LAI results were further employed for the statistics calculations.

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3. RESULTS

As a retrospective survey, there was no research protocol, therefore we report the incidental immune investigation as registered in the medical charts. The LAI mean was 44.8%, SD 26.9%, ranging from 0% to 94%, mode = 0% (appeared 14 times). There was a wide range of distribution of LAI results, suggesting that some patients had immunoreactivity against cobalt, while others do not. Some patients showed strong immunoreactivity during the *ex vivo* challenge test against cobalt, while others showed low or moderate immunoreactivity (see Fig 1.) That immunoreactivity theoretically could produce clinical symptoms when the patient contacts or ingests cobalt. As a preliminary descriptive finding, we cannot take a definitive conclusion about it. Controlled and prospective studies are needed to achieve the best knowledge of this theme.

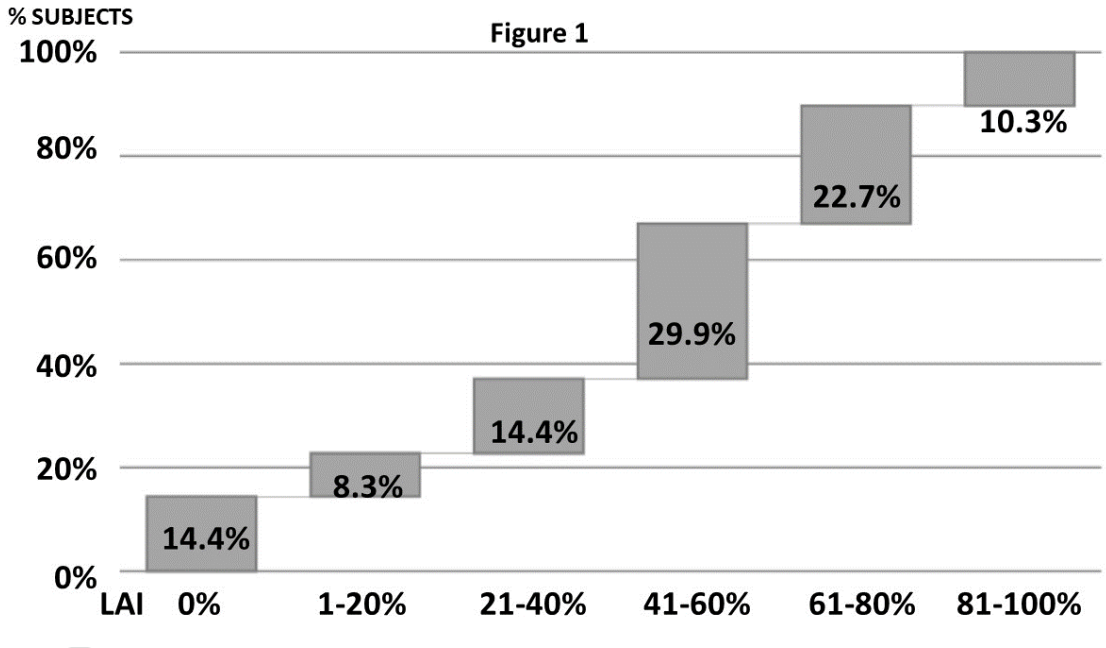

Fig. 1. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (LAI) results (x-axis %) of *ex vivo* $CoCl_2 \cdot 6H_2O$ challenges monitored by the Leukocyte Adherence Inhibition Test (LAIT), according to the respective percentage of results over 97 tests (y-axis).

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4. DISCUSSION

After the introduction of the EU Nickel Directive regulating the presence of nickel in close-contact alloys of inexpensive jewelry, most attention was turned toward the emergent sensitization against cobalt alloys [58]. Cobalt ranks eighth (8.4%) as the most frequent cause of ACD diagnosed by the North American Standard Patch Test Battery [59]. As a public health issue, legislators are essaying efforts to promote the regulation of implantable medical devices that should be designed and manufactured in a way to minimize the risks associated with the aging of the materials [60]. Meanwhile, bioengineering is searching for new alloys free of nickel and cobalt to avoid the inconvenience of the liberation of these metals from metallic implants [61].

The Patch Test is the more convenient clinical tool to investigate the delayed hypersensitivity reactions produced by metals [62, 63]. However, the Patch Test may not be as sensitive, or as reliable as the Oral Provocation Test, which can reveal hypersensitivity in patients not reactive to the Patch Test [64]. However, the risk of sensitization after these procedures is a concern [65]. Studies had shown that delayed hypersensitivity is associated with a T-cell cytokine response [66, 67]. However, the Lymphocyte Transformation Test (LTT) alone is not the best choice to exclude a hypersensitivity reaction, because these reactions are mostly induced by cytokines secreted by macrophages rather than lymphocytes [68, 69]. Consequently, the presence of macrophages amplified the *ex vivo* stimulation of T-cells [70]. The Leukocyte Migration Inhibition Test was one of the best attempts to diagnose specific immunoreactivity to haptens in patients with ACD, creating new opportunities to *ex vivo* challenges in the diagnosis of metal hypersensitivities [71-74].

The causal agents of ACD may be diagnosed by the lymphocyte stimulation test associated with an evaluation of the cytokine produced by challenged cultures of peripheral blood mononuclear cells (PBMC) [75]. Cobalt hypersensitivity is a lymphocyte-mediated Type IV Gell & Coombs delayed reaction that occurs after contact with a sensitized individual [76]. Cobalt works as a hapten, a low-molecular-weight cation able to be covalently bound to a heavier carrier [77]. Most of the knowledge about hapten-induced contact hypersensitivity is derived from murine studies demonstrating apoptosis of keratinocytes after activation of $CD8^+$ and $CD4^+$ type 1 T cells and liberation of proinflammatory cytokines [78]. To become a full antigen, cobalt must bind to proteins before being able to interact with immune cells inducing antigen presentation and differentiation of T effector cells able to develop hypersensitivity reactions [79]. As a hapten, cobalt may also link to host proteins, mainly the Human Serum Albumin inducing the production of antibodies against the hapten carrier complex, developing (IgE and Non-IgE) antibody-mediated hypersensitivities, classified as Types I, II, and III by Gell & Coombs [80].

For 5 years, our Institute has employed *ex vivo* challenge tests monitored by the Leukocyte Adherence Inhibition Test (LAIT) as a diagnostic complement for patients with a strong clinical suspicion of cobalt hypersensitivity and negative Patch Tests. The LAIT is a test equivalent to the Leukocyte Migration Inhibition Test, exploiting a common physiology but is easier to perform and standardize [81].

Recently, we published a similar paper considering the use of the LAIT to evaluate immunoreactivity against nickel in patients with nickel hypersensitivity [82]. As an unspecific technic to observe the final behavior of leukocytes (adherence inhibition under contact with a previously memorized antigen), the LAIT does not demonstrate the activation of any particular immune pathway, but just the presence of immune memory and immunoreactivity developed after a previous contact [83, 84]. The LAIT may be seen just as a triage test to provide a clue over the presence, importance, or absence of immunoreactivity against a particular antigen or allergen [85]. LAIT works as a final overview of an immune response common to several immune pathways [86].

Our preliminary retrospective survey has demonstrated in a group of allergic patients a great range of results against the *ex vivo* challenge against cobalt, suggesting that some patients had already an immunological experience with this hapten. The extent of this immune experience and the frequency of the contact with this hapten would determine if this is really a trigger of clinical symptoms. The best tool to diagnose this is the *in vivo* provocation tests, supervised by experienced health professionals. Meanwhile, the LAIT may work just as a quick and inexpensive pre-test to select worthwhile antigens to proceed with the more elaborated *in vivo* provocations. More studies with prospective larger double-blind cohorts are in need to validate the hypothesis that the LAIT can potentially be used as a diagnostic tool to demonstrate cobalt immunoreactivity.

5. CONCLUSION

Our preliminary results support the fact that the LAIT performed with $CoCl_2 \cdot 6H_2O$ may differentiate diverse degrees of *ex vivo* immunoreactivity against cobalt in allergic patients.

CONSENT

It is not applicable.

ETHICAL APPROVALS

As per international standards written ethical approval has been collected and preserved by the authors.

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