Contribution of Leukocyte AdherenceInhibition Test in the evaluation of Non–IgEmediatedimmunoreactivityagainst benzoicacid in allergic patients.

ABSTRACT

Aim: To evaluate the potential of the Leukocyte Adherence Inhibition Test (LAIT) to discriminate non–IgE-mediated immunoreactivity against benzoic acid in patients with non–IgE-mediated allergic phenotypes.
Study Design: We retrospectively examined the medical charts of 100 patients diagnosed with allergic rhinitis, allergic bronchitis, asthma, sinus headache, atopic dermatitis, and/or urticaria with clinical suspicion of non–IgE-mediatedbenzoic acid hypersensitivity who were investigated with *ex vivo* challenge test monitored by LAIT against benzoic acid.
Place and Duration of Study: Instituto Alergoimuno de Americana – São Paulo – Brazil – between January 2018 and March 2024.
Methodology The percentage of Leukocyte Adherence Inhibition (LAI) promoted by the *ex vivo* challenges against 1 mg/mL benzoic acid was distributed in ranges through a cascade distribution chart to outline the variability of the results.
Results: The LAI ranged from 0% to 97%; the Mean was 41.1%; the Median was 40.5%; the Standard Deviation was 24.2%; the Mode was 0 and 59 (each appeared four times). The cascade distribution demonstrates a wide distribution of LAI results. This extensive distribution of LAI results suggests that some patients had mild, moderate, or severe non–IgE-mediated immunoreactivity against benzoic acid, while others did not present any immunoreactivity against it.
Conclusion: Our preliminary results support that the LAIT performed with benzoic acid may discriminate diverse degrees of *ex vivo* immunoreactivity in patients suffering from diversified allergic phenotypes.

Keywords: Allergy; Asthma; Atopic Dermatitis; Bronchitis; Diagnosis; Exposome-wide association study; Hypersensitivity; Leukocyte Adherence Inhibition Test; Non–IgE-mediated Immunoreactivity; Precision Medicine; Rhinitis; Sinus Headache; Urticaria.

1. INTRODUCTION

Benzoic acid is an organic compound formed by an aromatic ring and a carboxyl group[1]. Benzoic acid is a parent and a metabolic common pathway of a large group of structurally related substances (aromatic salts, alcohols,aldehydes, esters, and acetals) legally regulated to be used as additives to industrialized foods, cosmetics, and medicaments [2]. Used as an antimicrobial, the legally acceptable daily intake uppermost limit for benzoic acid (or the benzyl/benzoic moiety) is 5.0 mg/kg body weight [3]. There is evidence that benzyl benzoate is hydrolyzed to benzyl alcohol and benzoic acid; as well, benzyl alcohol and benzaldehyde suffer *in vivo* oxidation to benzoic acid [4]. In plants and animals, benzoic acid is produced endogenously through the phenylalanine–tyrosine pathway[5]. Several of its derivatives occur naturally in foods, such as fruits (apple, avocado, blackberry, blueberry, cherry, cranberry, melon, papaya, plum, raspberry, strawberry, tomato), vegetables (artichokes, asparagus, beans, cabbage, corn, leek, mushroom, potatoes), meats (beef, chicken, pork, shellfish), cheeses, teas and wines [6].

Food additives (among them benzoic acid) have long been described as sensitizer agents responsible for human allergic reactions [7]. A double-blinded provocation study done with benzoic acid produced objective reactions in 7% of patients with urticaria [8]. There is a report of a child who developed chronic cheilitis when ingesting daily benzoates-preserved industrialized foods [9]. There are reports of asthmatic patients who had a crisis of bronchospasm after the intake of benzoate-containing antiasthmatic medicines [10]. Reports of cross-reactivity among benzoates, azo dyes, and aspirin in patients with urticaria are also common [11].

Most provocation tests performed with benzoic acid do not elicit immediate reactions; instead, the reactions appear within 14 hours after the challenge [12]. The immunoreactivity elicited against benzoic acid is non–IgE-mediated and is not yet acknowledged explicitly among the recently classified hypersensitivity mechanisms [13]. The main knowledge about the mechanism of hypersensitivity against benzoate (and similar food additives) was brought by an ex vivo leukocyte challenge test determining the increasing sulfidoleukotriene production[14]. Since leukotrienes are known mediators of the leukocyte adherence inhibition phenomenon, we hypothesize that the employ of the Leukocyte Adherence Inhibition Test (LAIT) could help identify the endotype responsible for benzoic acid hypersensitivity [15-17].

We routinely employ the LAIT in our facilities to evaluate non–IgE-mediated immunoreactivity against suspected allergens, previously engaging in exhaustive provocation tests[18-24]. To evaluate the potential of the LAIT to discriminate non–IgE-mediated immunoreactivity against benzoic acid, we retrospectively compiled the electronic medical charts of patients with non–IgE-mediated allergic rhinitis, allergic bronchitis, asthma, sinus headache, atopic dermatitis, and/or urticaria who were investigated with this procedure.

The present study hypothesizes that the LAIT may differentiate diverse degrees of immunoreactivity against benzoic acid among patients suffering from allergic phenotypes.

2. MATERIALS AND METHODS

2.1 Subjects

After receiving Institutional Review Board approval from the Instituto Alergoimuno de Americana (Brazil; 03/2024), we proceeded with the electronic chart review of 8,500 outpatients who attended our facility from January 2018 to March 2024. A cohort of 100 outside patients had been submitted to an *ex vivo* allergen challenge test with benzoic acid 1mg/mL monitored with LAIT for presenting non–IgE-mediated allergic rhinitis, allergic bronchitis, asthma, sinus headache, atopic dermatitis, and/or urticaria.

This study did not include pregnant women, breastfeeding, and patients under biological and/or systemic anti-inflammatory therapy (corticoids, cyclosporin). The cohort counted 30 males; mean age 41.9 years; SD 20.4 years; range 2 to 90 years; median 43 years; modes = 26; 28; 43; 43 and 53 (each appeared four times); geometric mean = 34.4 years. This procedure was offered to patients with clinical suspicion of benzoate hypersensitivity who demonstrated a non-reactive or inconclusive skin test against benzoic acid [25].

2.2 *Ex vivo* Investigation: Leukocyte Adherence Inhibition Test

We performed the LAIT as previously described [26-34]. Shortly, each donor's fresh plasma was divided into two parts and used in paralleled *ex vivo* challenging tests with benzoic acid 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 benzoic acid (10µL of a solution with 1mg/mL and pH 7.5) or without benzoic acid (when used as control). 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. 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.

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 LAI ranged from 0% to 97%; the Mean was 41.1%; the Median was 40.5%; the Standard Deviation was 24.2%; the Mode was 0 and 59 (each appeared four times).

The cascade distribution demonstrates a wide range of distribution of LAI results (Fig.1). Four patients ignored the presence of the allergen on the plasma and presented no inhibition of leukocyte adherence (LAI = 0%) after contact with benzoic acid (4% of the tests). Some patients showed low or moderate immunoreactivity during the *ex vivo* challenge test. In contrast, others displayed strong immunoreactivity, which could possibly reflect the participation of benzoic acid in a theoreticalnon–IgE-mediated hypersensitivity condition to be further corroborated by *in vivo* provocation tests.

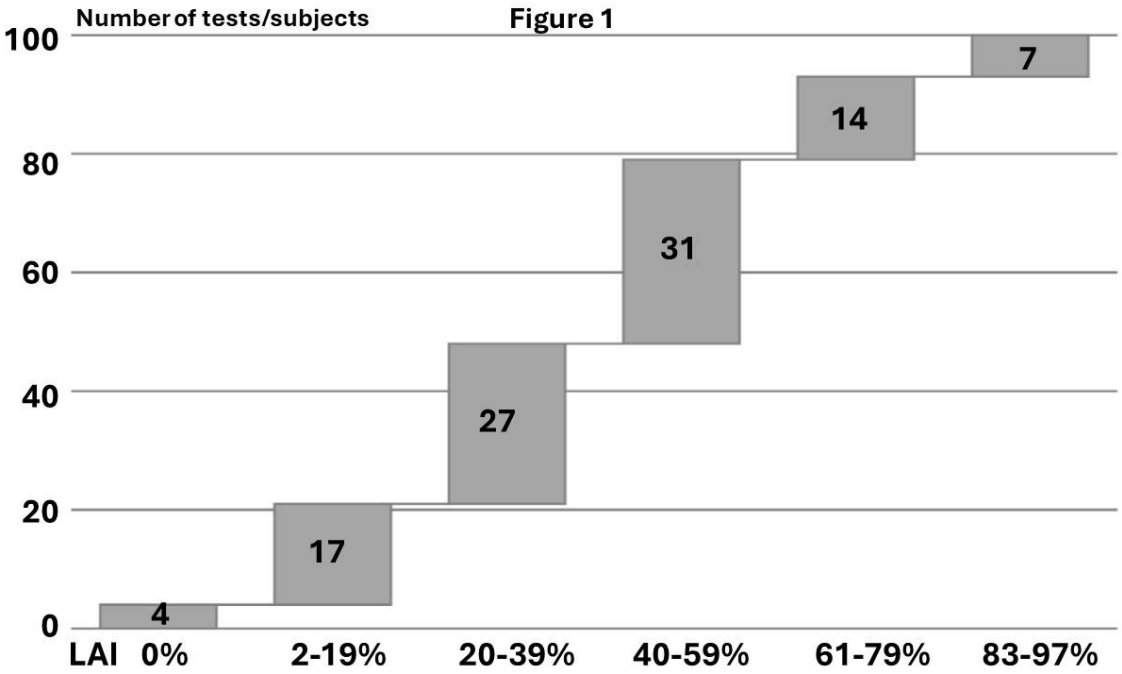

Fig. 1. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (LAI) results (x-axis %) of *ex vivo*benzoic acid challenges monitored by the Leukocyte Adherence Inhibition Test (LAIT), according to the respective number of outcomes over 100 tests/subjects (y-axis).

4. DISCUSSION

The non-IgE-mediated hypersensitivities are characterized by a challenging technical diagnosis due to the lack of standardized immunoassays[35]. Diagnosing these conditions among allergic patients is based on individual laborious medical work accomplishing anamnesis, cutaneous tests, and challenge *in vivo*provocation tests performed after meticulous exclusion diets [36].

As an increaser of the sulfidoleukotriene production, hypersensitivity to benzoic acid may be, in a certain way, similar (and potentially an enhancer) of the hypersensitivity produced by non-steroidal anti-inflammatory drugs (NSAIDs).The principal pharmacological action of NSAIDs is the inhibition of the cyclooxygenase enzymes, which catalyze the synthesis of prostaglandins and thromboxanes[37]. Cyclooxygenase enzymes catalyze the conversion of arachidonic acid released from the cellular membrane by cytosolic phospholipases activated by nociceptive mechanisms [38]. The cyclooxygenases and the lipoxygenases oxidize the arachidonic acid liberated into the cytosol.

The cyclooxygenases pathway generates pro-inflammatory autacoids such as prostaglandins and thromboxanes. The lipoxygenase pathway generates leukotrienes [39]. The pharmacologic inhibition of the cyclooxygenases increases the lipoxygenases' activity, increasing the leukotrienes' production. Any substance that (pharmacologically or immunologically) increases the production of leukotrienes affects the autacoid balance, adversely producing allergic symptoms.

The LAIT theoretically explores every immune pathway as an ex vivo challenge test with a viable leukocyte buffy coat, allowing the interaction of all immune-circulating participants[40]. However, as an observant of the final phenomenon, the LAIT does not indicate which pathways were involved in inhibiting the adherence (or increasingthe production of leukotrienes), whether pharmacological or immunological [41-44]. As proposed by the exposome-wide association study, the LAIT also configures itself as an exposome measurement,qualifying itself as an immune marker of the contact and the response to a specific antigen instead of being associated with a specific phenotype [45].

This preliminary retrospective survey demonstrated an extensive range of results from the *ex vivo* challenge test with benzoic acid monitored by LAIT in a cohort of patients with various allergic phenotypes.We routinely employ the LAIT as a complementary triage test to select worthwhile antigens to proceed with more laborious *in vivo* provocation tests when the specific IgE is undetectable. None of our patients presented an exclusive reaction to benzoic acid. Every patient was simultaneously tested with protein allergens (mites, fungi, food allergens), demonstrating positive results for some of them. Our results may suggest that allergic patients may impair their symptoms by a pharmacological or animmune additional action of benzoic acid over the hypersensitivity response.

5. CONCLUSION

Our preliminary results show that the LAIT may differentiate diverse degrees of *ex vivo*immunoreactivity against benzoic acid in patients clinically diagnosed with non–IgE-mediated allergies. The propaedeutic meaning of these results, however, must be established. More studies with prospective larger double-blind cohorts need to evaluate the potential contribution of LAIT for the etiologic diagnosis of patients suspected of symptomatic hypersensitivity against benzoic acid and other similar food, cosmetic, and pharmaceutical additives.

CONSENT

As a retrospective survey of results recorded *incognito*, consent was given collectively by the institution's ethics committee following the principles of the Declaration of Helsinki[46].

ETHICAL APPROVALS

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

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Abbreviations:
LAI: Leukocyte Adherence Inhibition
LAIT: Leukocyte Adherence Inhibition Test