Original Research Article

Endotyping Cellular and Humoral Immunoreactivity againstTartrazine in Allergic Patients. A Retrospective Study.

ABSTRACT

Background: Several publications report that tartrazine is responsible for IgE-mediated and non–IgE-mediated hypersensitivity reactions. There is no standardized lab exam to endotype non–IgE-mediated immunoreactivity against tartrazine besides *in vivo* provocation tests.
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 tartrazine in patientsclinically diagnosed with non–IgE-mediated allergic phenotypes associated with various non-IgE-mediated allergic conditions.
Study Design: We retrospectively examined the medical charts of two cohorts of patients diagnosed with the aforementioned allergic phenotypes with clinical suspicion oftartrazine hypersensitivity, who were investigated with the help of TTP (first cohort) or *ex vivo* challenge tests monitored by LAIT (second cohort) against tartrazine.
Methodology: The registered results of the semi-quantitative serum TTP against 1 mg/mL tartrazine 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 tartrazine solution were distributed in ranges through a cascade distribution chart to outline the variability of results inside the second cohort. The statistical characteristics of these cohorts were calculated.
Results: Most positive TTP results concentrated on the higher dilutions. The mean was estimated at 1:290; the standard deviation was estimated at 1:200.The LAI ranged from 0% to 88%. The mean was 35.4%; the standard deviation was 24,7%. The cascade distribution graph demonstrates that the LAIT distribution is mainly over the negative and weaker results.
Conclusion: Our preliminary results support that the TTP and LAIT performed with 1 mg/mL tartrazine solution may discriminate diverse humoral and cellular immunoreactivity degrees in patients suffering from severalnon–IgE-mediated allergic conditions, encouragingfurther prospective studies and validation.

Keywords: Asthma; Rhinitis; Endotype; Hypersensitivity; Leukocyte Adherence Inhibition Test; Precipitins; Tartrazine; Urticaria.

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

1. INTRODUCTION

Tartrazine is a tastelesssynthetic yellow-lemon water-soluble aromatic mono-azo dyeused as itssodium salt, potassium salt, calcium salt, and aluminum lake[1]. Azo dyes (organic compounds bearing the functional group R−N=N−R′ where R and R′ are aryl groups) such as tartrazine, sunset

yellow, and carmoisineare additivesaggregated to provide colorto medicines and processed foods in products such ascanned vegetables, chewing gum, frankfurters,macaroni, soft drinks, spaghetti,bread, butter, cheese, concentrated fruitjuices, ice cream, jellies, tomato ketchup,jam, candies, pickles, and others, regardless any intrinsic nutrition value, preservative activity or health benefit[2, 3].

Tartrazine maybe used alone or associated with aluminum lakesor blue colorants to produce green shades [1, 4]. Tartrazine is also known by the codes: E102, INS 102, CI 19140, FD&C Yellow 5, Yellow 5 Lake, Acid Yellow 23, Food Yellow 4, and trisodium 1-(4-sulfonatophenyl)-4-(4-sulfonatophenylazo)-5-pyrazolone-3-carboxylate [5].

The European Food Safety Authority Panel calculated a theoretical maximum daily exposure to tartrazine of 8.1 mg/kg/day for adults and13.1 mg/kg/day for children, establishing an Acceptable Daily Intake (ADI) of 7.5 mg/kg/day[6]. However, these limits do not represent enough environmental safety since they can damage aquatic fauna such as the freshwater zebrafish (*Danio rerio*) [7].

Tartrazine has been known to cause allergic reactions since 1958[8]. The extensive clinical spectrum of tartrazine hypersensitivity includes urticaria, angioedema, anaphylactic shock, asthmatic bronchitis, rhinitis, throat tickle, cough, vasculitis (purpura), and contact dermatitis [9-14]. A patient with textile contact dermatitis after using a yellow cloth was diagnosed with the help of a skin contact test with tartrazine [15].

The fact that tartrazine is a drug additive with no proper function besides coloring pills and solutions makes it one of the first aims to diagnose when facing allergic conditions elicited by colored medications such as antihistamines and steroids used to treat allergies and asthma [16].

Tartrazine belongs to a select group of aromatic substances (aspirin, salicylates, sulfites, benzoates, and azo dyes) known to produce non–IgE-mediated “allergic-like” dose-dependent symptoms such as urticaria and bronchospasm at remarkably similar molar doses in allergic patients submitted to progressive oral challenges [17-19]. These reactions appear to have a familiar incidence, presenting cross-reactivity among these substances [20].

Tartrazine hypersensitivity has been associated with aspirin sensitivity; however, the mechanism is obscure since aspirin inhibits the cyclooxygenases responsible for producing prostaglandins, while tartrazine does not have this effect [21, 22]. A multicenter study performed with patients with aspirin-induced asthma found a frequency of 2.6% of patients with tartrazine hypersensitivity [23].

Due to tartrazine hypersensitivity, there is a worldwide concern to substitute tartrazine from medicines and industrialized food for natural colorants such as curcumin (E100), riboflavin (E101),beta-carotene (E160a), annatto(E160b), or other chemical dyes such as quinoline yellow (E104), or yellow iron oxide (E172) [24, 25].

Azo dyes are relatively small compounds that cannot interact alone with antibodies, so in hypersensitivity reactions, they function as haptens, usually linked to complex proteins, such as albumins [26]. Commercial anti-tartrazine polyclonal antibodies are usually produced for research purposes by conjugating tartrazine with ovalbumin or allophycocyanin[27]. Although one can find commercial lab kits to detect specific IgE against conjugated tartrazine, antibodies of the IgE class are unlikely to mediate tartrazine hypersensitivity[28].

The primary laboratory marker of patients reacting to dyes is eosinophilia [29]. A radioimmunoassay inhibition assaywas designed to detect anti-tartrazine IgD and IgE antibodies. However, patients diagnosed with tartrazine hypersensitivity could be distinguished from controls only by their specific IgD, not by their specific IgE antibodies against tartrazine [30].

There is not yet any reliable routine lab exam to quantify immunoreactivity against tartrazine, and the clinical diagnosis of hypersensitivity to tartrazine is founded chiefly on *in vivo* tests based on oral or cutaneous provocations.

Cellular immunoreactivity against tartrazine (and other food additives) has already been demonstrated by *ex vivo* challenging tests monitored by the granulocytic myeloperoxidase release reaction [31]. *Ex vivo* challenges employing leukocytes to detect immunoreactivity against tartrazine were also already monitored by sulfidoleukotriene production in allergic patients [32].

The direct effect of tartrazine on lymphocytes is suggested by cytotoxic experiments employing *ex vivo* experiments employing human lymphocytes [33]. *Ex vivo* studies in cultured human leukocytes demonstrated that tartrazine at70 µg/mL induces DNA damage, suggesting a genotoxic potential[34].

The Leukocyte Adherence Inhibition Test (LAIT) and the Tube Titration of Precipitins (TTP) are performed in our facilities as triage tests to identify immunoreactivity against suspected allergens executedbefore the performance of more exhaustive *in vivo* provocation tests [35-41]. The present

study hypothesizes that LAIT and TTP may differentiate endotypes and degrees of immunoreactivity against tartrazine among patients suffering from common allergic phenotypes.

To evaluate the potential of the LAIT and the TTP to discriminate humoral and cellular immunoreactivity against tartrazine, we retrospectively compiled the electronic medical charts of patients clinically diagnosed with non–IgE-mediated allergic phenotypes associated with chronic and/or recurrent conditions such as rhinitis, sinusitis, conjunctivitis, bronchitis, allergic contact dermatitis, intrinsic atopic dermatitis, urticaria systemic anaphylactic reactions and/or gastrointestinal disorders who were investigated with these procedures.

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2. MATERIALS AND METHODS

2.1 Subjects

After receiving Institutional Review Board approval from the Instituto Alergoimuno de Americana (Brazil; 08/2024), we reviewed the electronic chart of 9,500 outpatients who attended our facility from January 2018 to October 2024.

The first cohort (TTP cohort) consisted of 100 outside patientsclinically diagnosed with non–IgE-mediated allergic phenotypes associated with chronic and/or recurrent conditions such as rhinitis, sinusitis, conjunctivitis, bronchitis, allergic contact dermatitis, intrinsic atopic dermatitis, urticaria systemic anaphylactic reactions and/or gastrointestinal disorders. These patients had been submitted to TTP with 1 mg/mL of tartrazine solution.

The TTP cohort counted 23 males and 77 females; mean age 35,5 years; SD 21.8 years; range 1 to 79 years; median 35 years; modes = 9, 27, 42, 43, and 69 (each appeared four times); geometric mean = 25.3 years.

The second cohort(LAIT cohort) consisted of 100 outside patientsclinically diagnosed with non–IgE-mediated allergic phenotypes associated with chronic and/or recurrent conditions such as rhinitis, sinusitis, conjunctivitis, bronchitis, allergic contact dermatitis, intrinsic atopic dermatitis, urticaria systemic anaphylactic reactions and/or gastrointestinal disorders.These patients had been submitted to an *ex vivo* allergen challenge test with tartrazine solution 1mg/mL monitored with LAIT.

The LAIT cohort counted 36 males and 64 females; mean age 40 years; SD 21.2 years; range 4 to 90 years; median 38 years; mode = 22 (appeared four times); geometric mean = 33.2 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 tartrazine hypersensitivity who demonstrated a non-detectable specific IgE against tartrazine and a non-reactive or inconclusive skin test done withtartrazine1 mg/mL solution [42].

2.2 Tartrazine solution

The tartrazine solution was prepared with powdered tartrazine 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 [43-52]. Shortly, each donor's fresh plasma was divided into two parts and used in parallel *ex vivo* challenging tests with tartrazine acetate solution 1 mg/mL and the unchallenged plasma assay. We collected 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 tartrazine solution (10µL of a solution with 1mg/mL) or without tartrazine 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 (phosphate-buffered saline)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 tartrazine solution was performed in a transparent vitreous tube array[53-55]. 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 tubeswere examined,and the titers (the highest dilution factor that yields a positive reading) were recorded [56].

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 distribution range of TTP results. There was one negative result, while more positive results concentrated on the higher dilutions (Fig 1). The mean was estimated at 1:290; the median was 1:256; the standard deviation was estimated at 1:200; the mode was 1:512 (appeared 42 times). All Sia tests were negative.

The LAI ranged from 0% to 88%. The mean was 35.4%; the median was 36.5%; the standard deviation was 24,7%; the mode was 0% (appeared nineteen times). The cascade distribution graph demonstrates distribution mostly over the negative and weaker results (Fig.2). Nineteen patients (19%) ignored the allergen, presenting no inhibition of leukocyte adherence (LAI = 0%) after contact of the plasma with the tartrazine solution. Some patients showed low or moderate immunoreactivity during the *ex vivo* challenge test, while most displayed strong immunoreactivity, suggesting tartrazine's participation in the hypersensitivity condition.

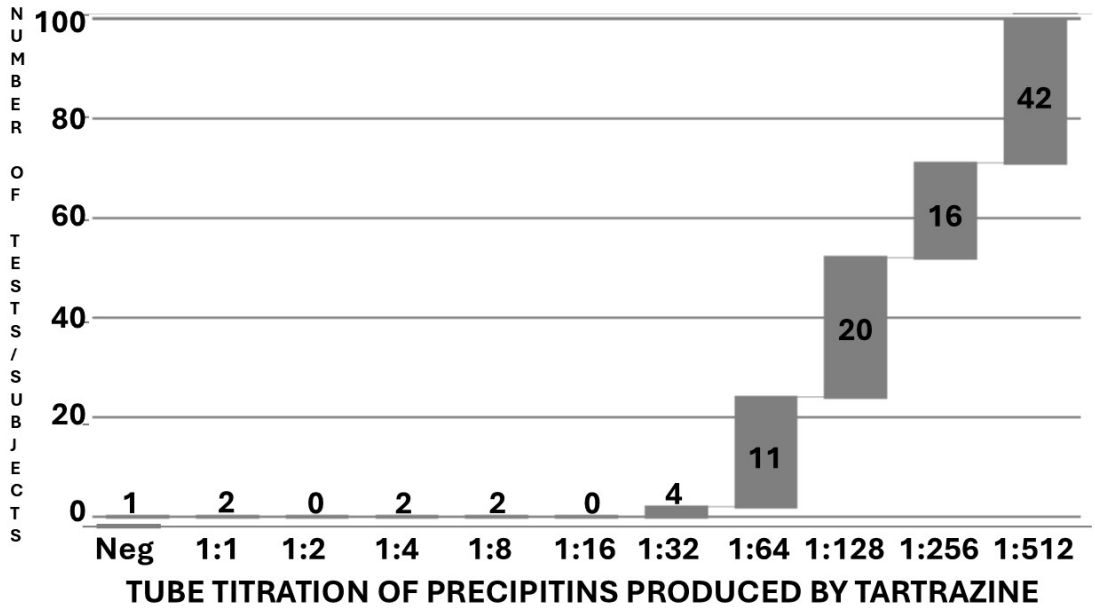

Fig. 1. Cascade distribution chart of the Tube Titration of Precipitins (TTP on the x-axis) resulting from the tartrazine solution against the serum of a cohort of 100 tests/subjects (y-axis).

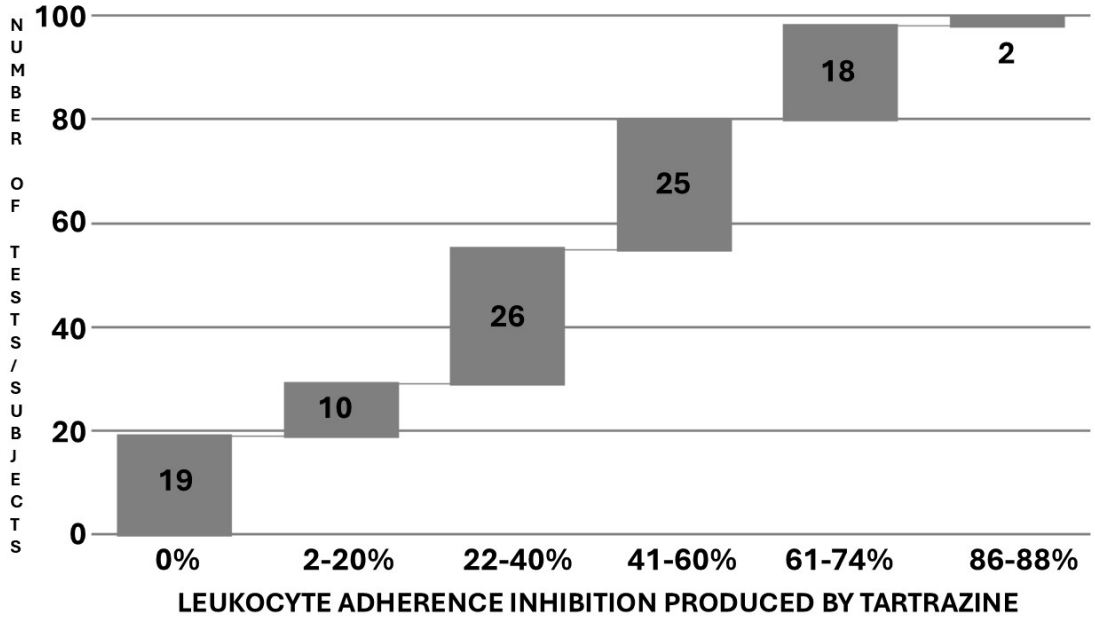

Fig. 2. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (LAI) results (x-axis %) of *ex vivo*tartrazine solution monitored by the Leukocyte Adherence Inhibition Test (LAIT), according to the respective number of outcomes over a cohort with 100 tests/subjects (y-axis).

4. DISCUSSION

To detect humoral and cellular immunoreactivitiesagainst tartrazine, we retrospectively compiled the data registered in an Excell® spreadsheet resulting from TTP and TIAL against tartrazine at our facilities. These triage tests are performed before the *in vivo* provocation tests when skin tests are unfeasible due to the patient's skin conditions.

The humoral and the cellular immunoreactivity profiles present divergent results. The humoral profile, represented by the TTP, presented the most results in the more diluted titrations, suggesting that most patients produced prominent levels of anti-tartrazine antibodies. However, the cellular profile, represented by the TIAL, presented most results in the negative or the weaker LAI results, suggesting a less determinant participation of the cellular immunoreactivity. The fact that the results were obtained from two cohorts and measured by different methodologies (quantitative and semi-quantitative) did not allow us to perform a paired t-test analysis.

The primary strategy of Personalized Medicine is to diagnose the endotypes responsible for the disease's phenotypes [57]. The former limited capacity to diagnose hypersensitivity had created in physicians the common idea that diagnosing a single hypersensitivity would be enough to treat their patients, conferring them a "mono-sensitization label". As medical knowledge and resources advance, more diagnoses are being performed, making physicians aware that poli-sensitization is more a rule than an exception. Panallergens responsible for cross-reactivity between allergens from diverse sources, such as tropomyosins or profilins, contribute to this awareness, allowing a more extensive comprehension of the allergic conditions [58, 59]. Cross-reactivity among azo dyes and similar preservatives, such as the sulfides, a group of inorganic salts added to processed foods and also naturally found in *Allium* spices and fermented beverages, is a large field for studies that must be explored to understand hypersensitivity conditions better, endotyping their mechanisms, and tailor the treatment of allergic patients [60, 61].

The great insight Gell and Coombs gave in the sixties resisted the test of time and now is amplified as a compass to understand the wide variety of hypersensitivity reactions that are still far from being completely elucidated [62, 63]. Endotyping underlying hypersensitivity mechanisms may help distinguish superimposable phenotypes presenting similar symptoms that may hamper establishing a precise diagnosis [64].

TTP and LAIT assays do not identify the exact immune mechanisms responsible for clinical hypersensitivity; instead, they are general immune markers of the humoral and cellular responses against allergens, quantifying an exposome measurement [65].

This retrospective proof of concept analysis demonstrated several degrees of humoral and cellular immunoreactivity, as demonstrated by TTP and LAIT against tartrazine in two cohorts of patients with several allergic conditions. None of the patients presented an exclusive reaction to tartrazine. Every patient was simultaneously evaluatedfor other suspected allergens, demonstrating positive and negative results. Our results suggest that tartrazine can elicit humoral and cellular immunoreactivity in allergic patients,theoretically able to impair their allergic symptoms.

5. LIMITATIONS

This study is a proof-of-concept retrospective analysis of data collected over six years. There was no protocol research, and the subject's data was 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

In vitro humoral assays and *ex vivo* cellular challenges can detect immunoreactivity against potentially lethal allergens without posing any risk for the patient and are occasionally employed in daily routine when conventional allergy diagnostic procedures are not elucidative or contra-indicated[66].Our preliminary results show that the TTP and LAIT may differentiate diverse degrees of

immunoreactivity against tartrazine 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 [67]. 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 tartrazine and other similar food processing additives [68].

7. FUTURE DIRECTIONS AND RECOMMENDATIONS FOR CLINICAL PRACTICE

The primary intended use of *in vitro* or *ex vivo* allergen challenges is to spare the patients from being submitted to exhaustive and dangerous *in vivo* challenge tests. Exploring the humoral and the cellular arms of immune systems, the TTP and TIAL alone or combined may represent,in the near future, a tool for allergists to construct an etiologic diagnosis from their patients, as well as determine the endotypes (mechanisms) of hypersensitivity, in order to choose more convenient and personalized therapies for them.

CONSENT

As a retrospective survey of results recorded *in cognito*, consent was given collectively by the institution's ethics committee following the principles of the Declaration of Helsinki [69].

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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