EndotypingNon–IgE-mediatedImmunoreactivity to Polyethylene Glycol:Implications for Allergic Patients

ABSTRACT

Background: Several publications report polyethylene glycols (PEGs) and PEGylated therapeutics as responsible for non–IgE-mediated allergic reactions. There is no standardized lab exam that can endotype (quantify the participation of these mechanisms inside each patient's pattern of symptoms) besides *in vivo* provocation tests.
Objective and aim: To evaluate the Tube Titration of Precipitins (TTP) and the Leukocyte Adherence Inhibition Test (LAIT) to discriminate and endotype non–IgE-mediated immunoreactivity against a PEGs solution in patients with non–IgE-mediated allergic phenotypes.
Research Design and research protocol: To retrospectively examine the medical charts of two cohorts (with 100 patients each) diagnosed with non–IgE-mediated allergic rhinitis, allergic bronchitis, asthma, allergic sinus headache, atopic dermatitis, and/or urticaria who were investigated with the help of TTP or *ex vivo* challenge tests monitored by LAIT against PEGs.
Methodology: We identifiedthe registered results of the semi-quantitative serum TTP against 1 mg/mL PEGs 4000 solution, which were distributed in ranges through a cascade distribution chart to outline the variability of the results inside the first cohort. The LAI done with the *ex vivo* challenges with 1 mg/mL PEGs 4000 solution were distributed in ranges through a cascade distribution chart The statistical characteristics of the two cohorts were calculated.
Results: The TTP showed a wide distribution range of results. The mean was estimated at 1:246; the median at 1:192; the standard deviation at 1:188. The LAIT showed a wide distribution range of results. The LAI ranged from 0% to 98%. The mean was 41.9%; the median was 45%; the standard deviation was 28.5%. The cascade distribution demonstrates a wide range of LAI results.
Conclusion: Some patients showed any or low immunoreactivity during the ex vivo challenge test, while most displayed moderate or strong immunoreactivity, which could reflect the participation of PEGs antibodies in a non–IgE-mediated hypersensitivity condition.

Keywords: Allergy; Asthma; Atopic Dermatitis; Bronchitis;COVID-19 vaccination; Diagnosis; Exposome-wide association study; Endotype; Hypersensitivity; Leukocyte Adherence Inhibition Test; Macrogol; Non–IgE-mediated Immunoreactivity; Precision Medicine; Polyethylene glycol; E1521; Rhinitis; Sinus Headache; Urticaria.

Abbreviations:
LAI: Leukocyte Adherence Inhibition
LAIT: Leukocyte Adherence Inhibition Test
TTP: Tube Titration of Precipitins
PEGs: Polyethylene glycol polymers

1. INTRODUCTION

For a long time, petroleum-derived polymeric products were discarded as tars. The first polymerized structures to be identified from tars were the polyethylene glycol polymers (PEGs) after continued condensation studies in 1860 [1]. PEGs are polymers of parental ether monomers such as ethylene glycol, ethylene oxide, or oxyethylene, commonly available as compounds together with their anionic or nonionic derivatives, in mixtures of different chain lengths polymers, indicated by their average molecular weight (200 to over 10,000 Da) [2].

PEGs are used as solvents and emulsifying agents in processed food, cosmetics, medicines, cleansing, and personal care products. They may also be involved in the production of fabrics, plastics, resins, papers, ceramics, glasses, rubber, metals, wood preservatives, et cetera [3]

"Chemically designated as alpha-hydro-omega-hydroxypoly(oxy-1,2-ethanediol) or polyethylene oxides, PEGs are regulated additives, allowed to be used as emulsifiers in industrialized food and food supplements (E 1521) and as plasticizers in film coating formulations for food supplement tablets and capsules" [4]. "PEGs are amphipathic substances soluble in water and in many organic solvents, including aliphatic ketones, alcohols, chloroform, glycol ethers, esters, and aromatic hydrocarbons. Acting as emulsifiers, they prevent the separation of unstable water/oil emulsions in industrialized food such as mayonnaise, dairy-based ice cream, frozen yogurts, margarine, blended spreads, cakes, pastries, and frozen desserts" [5]. When used as dietary emulsifiers, PEGs may increase intestinal permeability by interferingwiththe physical properties of the mucus layer, facilitating the growth of pro-inflammatory intestinal microbiota and contributing to the impairment of inflammatory intestinal diseases and food allergies [6, 7].

PEGs (also designated as macrogol in pharmacy) are used as vehicles for drugs and ointment bases, capsules, tablet and pill binders, suppositories, and liquid prescriptions, including parenteral, topical, ophthalmic, oral, and rectal preparations. They also prevent protein aggregation and inactivation of pharmaceutical proteins encapsulated in bioerodible polyester microspheres, acting as stabilizers [8]. PEGylation (or pegylation) is a process developed in the 1970s to covalent or non-covalently attach PEG polymers to molecules, such as drugs, therapeutic proteins, peptides, antibody fragments, and vesicles, then described as "PEGylated" [9]. "PEGylation changes the physical and chemical properties of the biomedical molecule, such as its conformation, electrostatic binding, and hydrophobicity, resulting in an improvement of the pharmacokinetic behavior of the drug, reducing coalescence, degradation and *in vivo* elimination of the active pharmacological principles" [10-12]. "PEGylated products can induce anti-PEG antibody production associated with type III complement-activated immunoreactivity" [13].

"Lower molecular weight PEGs are absorbed by the digestive tract and excreted in the urine; however, high molecular weight PEGs are not absorbed and form hydrogen bonds with water in the gastrointestinal tract, where they hydrate stools" [14]. Long-chain PEGs are laxative medications in isosmotic balanced electrolyte preparations [15]. PEGs are also used in topical dermatological preparations as penetration enhancers [16]. PEGs and their derivatives (laureth, ceteth, ceteareth, steareth, oleth, laurate, dilaurate, stearate, distearate) do not readily penetrate intact skin and are added to a great variety of cosmetic applications as surfactants, cleansing agents, emulsifiers, skin conditioners, and humectants. [17]. However, absorption through damaged skin of PEGs present in burn creams was associated with fatal intoxication in burned patients characterized by acute renal failure and metabolic acidosis with increased calcium, anion, and osmolar gap [18]. Allergic reactions due to PEGs used as excipients have already been reported [19].

Pharmaceutical excipients are a hidden potential cause of drug and vaccine hypersensitivity reactions [20]. Allergic reactions to PEGs were put into evidence, particularly after Pfizer-BioNTech® and Moderna® mRNA COVID-19 vaccines were approved for mass vaccination [21, 22].

Endotyping pharmaceutical excipient immunoreactivity has become a herculean task due to the variety and diversity of formulations, mainly after the appearance of the PEGylated lipid nanoparticle vaccines [23]. PEGs are the most popular nanomedicine components due to their chemical properties [24].

The IgE-mediated anaphylaxis elicited by PEGs excipients was the first and easiest endotype to diagnose [25, 26]. Skin and basophil activation tests are the most available assays to identify immediate and/or IgE-mediate allergy to PEGs [27]. However, intradermal skin tests performed with PEGs may produce immediate anaphylactic reactions, which makes the percutaneous route a more secure way to perform *in vivo* challenges [28]. At our facilities, we had performed hundreds of skin tests with PEGs employing the skin scrape test technique with no systemic reaction, despite dozens of positive wheal-and-flare local diagnostic reactions elicited by PEGs [29]. A Dual Cytometric Bead Assay (DCBA) was recently described for detecting anti-PEG IgG, IgM, and IgE in patient sera [30].

Besides the type I IgE-mediated hypersensitivity reaction, several other mechanisms have been proposed, such as IgG-mediated anaphylaxis (Complement-related or not) and direct mast cell activation [31]. The production of Anti-Poly(Ethylene Glycol) antibodies, besides provoking hypersensitivity reactions, can also be problematic when a PEGylated drug is administered, accelerating drug clearance and decreasing its therapeutic efficacy [32, 33].COVID-19 mRNA vaccination is associated with elevated levels of vaccine-induced anti-PEGs antibodies and increased

systemic reactogenicity [34]. PEGs excipients are also suspected to elicit life-threatening immune-induced organ-specific reactions such as acute pancreatitis, acute interstitial nephritis, and liver injury [35].

We routinely employ the Leukocyte Adherence Inhibition Test (LAIT) and the Tube Titration of Precipitins (TTP) in our facilities as a triage to evaluate non–IgE-mediated immunoreactivity against suspected allergens before the performance of more exhaustive *in vivo* provocation tests [36-42]. The present study hypothesizes that the LAIT and the TTP may differentiate diverse endotypes and degrees of immunoreactivity against PEGs among patients suffering from the most common allergic phenotypes. To demonstrate diversity in immune responses and to evaluate the potential of the LAIT and the research of precipitins to endotyping non–IgE-mediated immunoreactivity against PEGs, 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 these procedures into our institution.

2. MATERIALS AND METHODS

2.1 Subjects

After receiving Institutional Review Board approval from the Instituto Alergoimuno de Americana (Brazil; 05/2024), we reviewed the electronic chart of 9.000 outpatients who attended our facility from January 2018 to June 2024.

The sample selection was performed by retrieving the results from the electronic sheets.

A cohort of 100 outside patients had been submitted to TTP with PEGs solution 1mg/mL for presenting non–IgE-mediated allergic rhinitis, allergic bronchitis, asthma, allergic sinus headache, atopic dermatitis, and/or urticaria. This cohort counted 31 males; mean age 34.8 years; standard deviation (SD) 21.3 years; range 0 to 80 years; median 32.5 years; mode = 28 years (appeared five times).

A different cohort of 100 outside patients had been submitted to an *ex vivo* allergen challenge test with PEGs solution 1mg/mL monitored with LAIT for presenting non–IgE-mediated allergic rhinitis, allergic bronchitis, asthma, allergic sinus headache, atopic dermatitis, and/or urticaria. This cohort counted 27 males; mean age 39.9 years; SD 20.5 years; range 3 to 84 years; median 39.5 years; modes = 24 and 41 years (each appeared four times).

This study did not include patients under biological and/or systemic anti-inflammatory therapy. These procedures were offered to patients with clinical suspicion of PEGs' hypersensitivity who demonstrated a non-reactive or inconclusive allergic skin scrape test against PEGs' solution [29].

2.2 PEGs solution

The PEGs solution was prepared with powdered PEG 4000 (acquired from ACS Científica) diluted with isotonic saline solution at 1 mg/mL to perform the allergic skin scrape 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 [43-51]. Shortly, each donor's fresh plasma was divided into two parts and used in paralleled *ex vivo* challenging tests with PEGs 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 PEGs solution (10µL of a solution with 1mg/mL and pH 7.5) or without PEGs 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 calculating the adherence inhibition

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 PEGs solution was performed in a transparent vitreous tube[52]. 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 (PEGs solution 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, one of us examined the tubes, and the titers (the highest dilution factor that yields a positive visual reading) were recorded [53].

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 TTP showed a wide distribution range of results. There were two negative results. Most positive results concentrated on the higher dilutions (Fig 1). The mean was estimated at 1:246; the median was 1:192; the SD was estimated at 1:188; the mode was 1:512 (appeared 30 times). All Sia tests were negative.

The LAIT showed a wide distribution range of results. The LAI ranged from 0% to 98%. The mean was 41.9%; the median was 45%; the SD was 28.5%; the mode was 0% (appeared seven times). The cascade distribution demonstrates a wide range of distribution of LAI results (Fig.2). Six patients ignored the presence of the allergen on the plasma and presented no inhibition of leukocyte adherence (LAI = 0%) after contact with the PEGs extract (6% of the tests). Some patients showed any or low immunoreactivity during the *ex vivo* challenge test, while most displayed moderate or strong immunoreactivity, which could reflect the participation of PEGs antibodies in a non–IgE-mediated hypersensitivity condition.

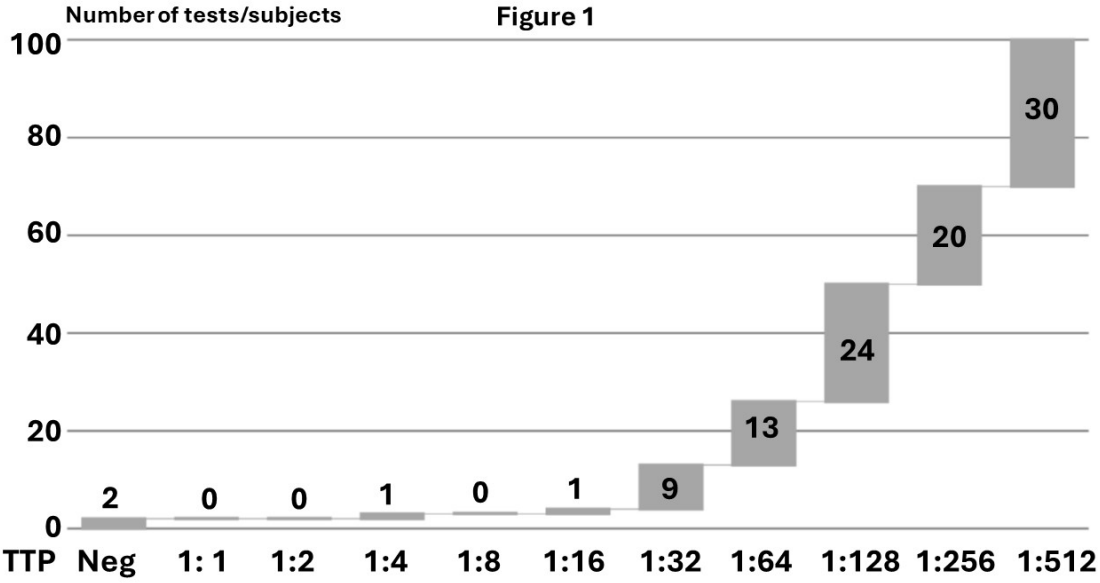

Fig. 1. Cascade distribution chart of the Tube Titration of Precipitins (TTP on the x-axis %) resulting from the PEGs solution against the serially diluted serum of a cohort of 100 tests/subjects (y-axis).

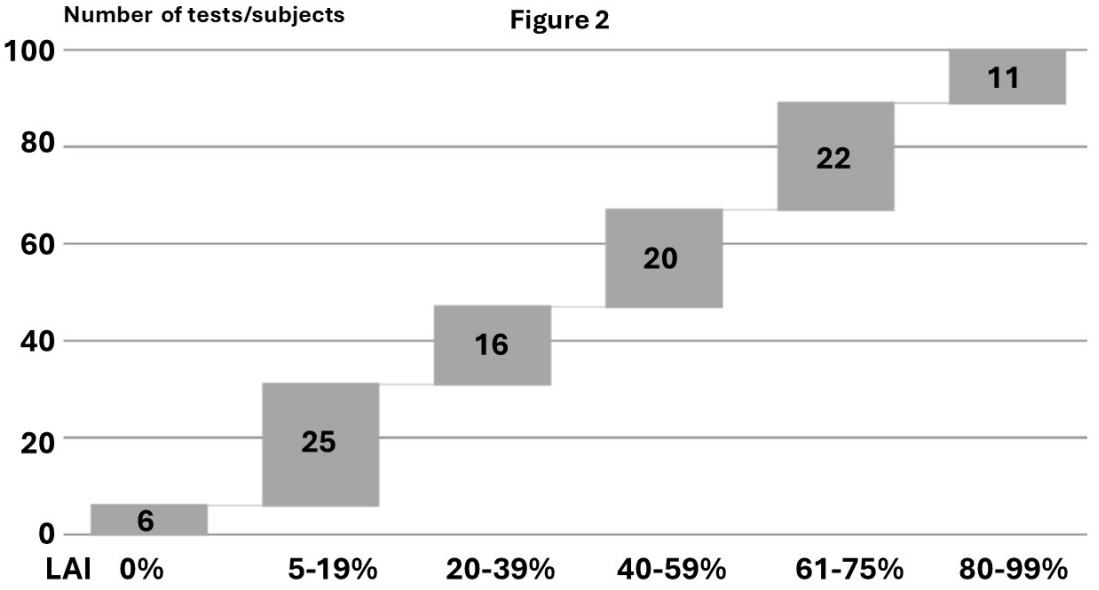

Fig. 2. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (LAI) results (x-axis %) of *ex vivo* PEGs solution monitored by the Leukocyte Adherence Inhibition Test (LAIT), according to the respective number of outcomes over a cohort with 100 tests/subjects (y-axis).

4. DISCUSSION

More and more patients are being diagnosed with PEG allergy as an increasing number of processed foods, cosmetics, household products, vaccines, and medicines add PEG to their composition [54]. The development of new PEGylated compounds is advancing, especially among biological immune therapeutics [55]. PEGs are the polymer of choice for the delivery of small drugs, proteins, oligonucleotides, and liposomes due to their biocompatibility and ability to improve pharmacokinetics;however, PEGsare a mixture of different polymers that can stimulate unwanted immunogenic reactions, leading to efforts to develop synthetic (monodisperse)more uniform PEGs [56].

PEG hypersensitivity is associated withmedicine-induced anaphylaxis, and PEGylated-drug reactions should prompt PEGs hypersensitivity investigations, especially by use of skin allergic tests with high molecular weight PEGs [57]. Diagnosing PEG allergy is challenging since the traditional allergy testing approaches are insufficient to demonstrate all kinds of hypersensitivity reactions [58]. Besides the IgE-mediated hypersensitivity reaction, the production of specific IgM and IgG antibodies and Complement activation were also described [59].Since a methodology for specific-IgE research has not yet been described, the feasibility of in vitro challenge tests, such as the Basophil Activation Test, is being studied [60]. Brand new allergic diagnostic techniques (such as performing tests with the help of PEGylated liposomes) are currently being designed to endotype PEGs allergies[61].

The precise diagnosis of the phenotypes and endotypes of hypersensitivities to PEGylated medications is paramount for prescribing a successful PEGs desensitization [62].

This retrospective compilation of our data showed a large distribution of results when we ascertained the results of TTP and TIAL to explore humoral and cellular immunoreactivity against PEGs. These immunoassays do not identify the exact 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.

The semi-quantitative study of the serological reactions through the titration of precipitins was the most basic laboratory exam upon which the fundamentals of Immunology were constructed [63]. Precipitating antibodies are classically associated with a robust immune humoral response against an antigen or allergen [64].The LAIT is an *ex vivo* challenge test performed with a viable leukocyte buffy coat that can theoretically explore most known immune pathways as it allows the interaction of all immune-circulating participants with the allergen [65]. Similarly, with the TTP, the LAIT did not indicate which pathways are involved in producing the immune cellular response demonstrated by the leukocyte adherence inhibition [66-69].

TTP and LAIT must be interpretedas humoral and cellular markers of the immune response after contact with a specific antigen, configuring themselves as techniques to endotype the exposome phenomenon, as proposed by the exposome-wide association study [70].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. None of our patients presented an exclusive reaction to PEGs. Every patient was simultaneously tested with several chemical and biological allergens, demonstrating positive results for some of them. Our clinical experience suggests that reactive allergic patients may impair their symptoms by an additional immunoreactivity against PEGs.

This preliminary retrospective survey demonstrated an extensiverange of results from the TTP and the *ex vivo* challenge test monitored by LAIT against PEGs in two cohorts of patients with various allergic symptoms. The preliminary results support that the TTP and LAIT performed with 1 mg/mL PEGs 4000 solution may discriminate diverse degrees of in vitro and ex vivo immunoreactivity in patients suffering from diversified allergic phenotypes. It is worth carrying out more in-depth studies to evaluate the usefulness of TTP and LAIT in endotyping non–IgE-mediated hypersensitivity to PEGs.

5. CONCLUSION

Our society is now flooded with chemical derivatives through industrialized foods, cosmetics, cleansing products, and medicines producing unknown immunoreactivity [71].Our preliminary results show that the LAIT and TTP may differentiate diverse degrees of immunoreactivity against PEGs in patients clinically diagnosed with non–IgE-mediated allergies. This methodology can provide a socioeconomic impact since the technology to perform TIAL and TTP is inexpensive and can be achieved in a single lab room attached to the clinical facility with minimum laboratory equipment. However, the propaedeutic meaning of these resultsand the possibility of interferents must be better

established[72]. Studies designed to establish diagnostic cutoffs focused on the quality-by-design approach with prospective larger double-blind cohorts need to evaluate the potential contribution of LAIT and TTP for endotyping immunoreactivity in patients suspected of symptomatic hypersensitivity against PEGs [73].

6. 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 comprehensiveand include patients' follow-up information. The lack of a research protocol implies the possibility of a bias produced by the physician's point of view, which indicated the exam (CEO) based on a clinical suspicion led by the anamnesis, physical examination, and *in vivo* provocation 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.

ETHICAL APPROVALS

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

Disclaimer (Artificial intelligence)

Authors 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 manuscripts.

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