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Assessment of Immunoreactivity against Therapeutic Options Employing the Leukocyte Adherence Inhibition Test as a Tool for Precision Medicine

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ABSTRACT

Background: **The Precision Medicine's approach employs the endotype concept as a central feature to personalize medical treatment. Individual immunoreactivity, alongside characteristics such as genetics, environment, and diet, is one of the factors that differentiates the therapeutic-driven endotypes.**

Objective: **To evaluate the opportunity of the Leukocyte Adherence Inhibition test to differentiate the immunoreactivity between two similar therapeutic agents employed on Allergen Immunotherapy.**

Methods: **Side by side Leukocyte Adherence Inhibitions tests were performed with ovalbumin and carbamylated ovalbumin on a population of 33 self-reported egg-allergic individuals.**

Results: **The results showed two endotypes inside the immune response of the studied groups: The first endotype was defined by the 16 individuals that presented a significant decrease in ovalbumin's immunoreactivity after carbamylation (mean of differences = 35%; p = 0.002). The second endotype was defined by 17 individuals that presented a significant increase in ovalbumin's immunoreactivity after carbamylation (mean of differences = 32%; p = 0.001).**

Conclusion: **The Leukocyte Adherence Inhibition test was able to differentiate two distinct immunoreactivity patterns when comparing two similar therapeutic agents suggesting, as proof of concept, a potential role to be employed as a Precision Medicine tool.**

Keywords: Allergoids, Leukocyte Adherence Inhibition test, Hypersensitivity, Precision Medicine.

Abbreviations:
LA: Leukocyte Adherence
LAR: Leukocyte Adherence Ratio
LAI: Leukocyte Adherence Inhibition
OVA: ovalbumin
cOVA: carbamylated ovalbumin
pOVA: phosphorylated OVA

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I. INTRODUCTION

Among the main causes of allergic symptoms that deserve a Precision Medicine approach are food allergies [1]-[5]. The Precision Medicine Initiative is a worldwide effort created to provide personalized care to patients based on the concept of the existence of different endotypes inside the larger phenotype groups as defined by modern Medicine [6]-[8]. The Precision Medicine approach also embraces the "complex endotype" concept, consisting of several sub-endotypes, instead of a single simplified endotype molecular mechanism [9]. Precision Medicine is mainly focused on therapeutic-driven endotypes, i.e. particular phenotype-subgroups that will better respond to a given therapy, instead of another [10]. The main concern is to increase efficacy and decrease collateral effects in the assumption that the peculiarities of each patient will produce diverse beneficial as well different collateral responses to the same therapeutic, as observed inside the whole population [11]. The development of Precision Medicine is based on the development of diagnostic assays to predict the specific response of each patient to a potential therapy, before the prescription of the treatment [12]. This concern, in the field of Allergology, has already originated the conception of more immunogenic and less allergenic alternatives to allergen-specific immunotherapy: the elaboration of modified allergens, the allergoids [13]. The use of allergoids for desensitization of allergic diseases has been reported since 1938 when Sledge *et al.* treated their hay fever patients sensitive to grass and ragweed with alum-precipitated pollen extracts, asserting that this modification provided better results and a significant reduction of "constitutional reactions" when compared with the natural extracts [14]. However, despite the alum-precipitated extracts could act better for some patients, some individuals presented allergic symptoms to the alum itself, characterizing a novel endotype among the patients belonging to this allergic phenotype [15]. Since then, dozens of techniques and adjuvants were described to decrease allergenicity and increase the immunogenicity of allergoids to provide a safer, more comfortable, personalized, and effective desensitization to allergic patients. One of the techniques employed to produce allergoids is protein carbamylation [16]. Protein carbamylation is a phenomenon that occurs naturally inside urea producers' organisms. The electrophilic species in equilibrium with urea: cyanate and isocyanate can react with lysine residues of proteins in an irreversible posttranslational modification, producing a carbamoyl group [17]. This spontaneous carbamylation occurring into physiologic systems (mainly in individuals with the uremic syndrome) may be assayed by colorimetric methods and is also considered a signal of molecular aging, related to degenerative disorders such as atherosclerosis and rheumatic inflammation [18], [19]. Minor changes in tridimensional conformation, such as the produced by carbamylation, can alter immunoreactivity, which depends on the antigen's conformational epitope [20]. This modification done *in vitro* can impair the binding of a reaginic antibody to the natural antigen, converting the desensitization's allergen into an allergoid [21]. Theoretically, this may not necessarily alter its tolerogenic capacity, which depends on the linear epitopes produced by Dendritic Cell's intracellular digestion that are presented to the naive T Cells into the MHC class II context [22].

As a proof of concept, we compared the immunoreactivity of self-reported egg-allergic individuals against a natural allergen: the ovalbumin (Gal d 2) and its corresponding carbamylated allergoid. Ovalbumin is the main egg white hen's protein with an approximate molecular weight of 43 kDa and consists of 385 amino acids single chain, 19 of which are lysine residues [23]. Meta-analyses comparing diverse diagnostic criteria (self-reported, skin tests, specific-IgE, and double-blind placebo-controlled provocation tests (DBPCPT) estimated that hypersensitivity to the egg is among the three most common causes of food allergic reactions [24]. The prevalence of food allergic reactions is relatively greater when the self-reported method is employed, compared with the objective diagnostic methods, but interestingly, the prevalence's ranking among the eliciting allergens is consistently maintained when comparing the objective and the self-reported criteria. This is understandable when we consider that allergy may be mediated by immune mechanisms not dependent on IgE and that food hypersensitivity reactions may also be dependent on thresholds, drugs, and physiologic stimuli, not always employed by *in vivo* provocation's tests [25]-[28]. Ovalbumin is not the most allergenic protein of hen's egg (the most allergenic is ovomucoid), but it is the most abundant and accessible protein in albumen [29]. Additionally, albumins are an interesting model to study immunoreactivity because they are produced by the human liver (human serum albumin), are present in significant amounts in human blood, and can be found in several edible animals and plants with a high degree of conservative evolution, resulting in both the possibilities of natural immunotolerance or cross-reactive hypersensitivity [30], [31]. Additionally, ovalbumin is also a very well-studied experimental inducer of tolerogenic Dendritic Cells [32]-[34]. One of the main functions of the albumins are the internal transport of ligands due to their conformational flexibility, another factor to alter their allergenicity [35]. There are three subclasses in the purified ovalbumin, according to their phosphate group content (two, one, or none) [36]. Four interchangeable secondary structures are found in an ovalbumin sample: alpha-helix, beta-sheet, random coil, and beta-turns. The heating stimulates denaturation, decreasing the alpha-helices and increasing the aggregational beta-sheets (beta-aggregation) [37]. These characteristics predict a broad specter of possibilities for immune reactions and sensitization mechanisms. To compare the general immunoreactivity against this complex allergen and its correspondent carbamylated allergoid, we used the Leukocyte Adherence Inhibition Test (LAI test) employing heparinized plasma of human patients with self-reported egg allergy. Leukocytes are naturally programmed to adhere to glass in physiologic conditions, which can be easily observed with an optical microscope and a glass surface hemocytometer chamber [38]. When functionally activated, they lose this capability, which allowed Halliday to design an antigen-specific *ex vivo* challenge named Leukocyte Adherence Inhibition test (LAI test) [39]. Challenged by specific antigens, leukocytes release paracrine soluble factors that interfere with glass adherence of nearby leukocytes, a phenomenon that can be quantified with a concomitant assay done with unchallenged plasma. Several immune mechanisms can produce this phenomenon, which seems to be just the final indicator of the antigen-specific leukocyte

activation or, in other words, the immunoreactivity [40]-[43].

II. Methods

A. Subjects

After receiving Institutional Review Board approval, from the Instituto Alergoimuno de Americana (Brazil), 33 consecutive outpatients complaining of self-reported challenge-proofed clinical symptoms compatible with hen's egg allergy, were invited, with informed consent formularies, to voluntarily provide blood samples to perform *ex vivo* challenge tests, according to the principles of Helsinki and the International Committee of Medical Journals Editors requirements of privacy. All patients had non-detectable specific IgE to ovalbumin and inconclusive skin tests (unreactive histamine controls) [44]. The study was purely descriptive and did not interfere with the patient's treatment or the assistant physician's diagnosis. All relevant and mandatory laboratory health and safety measures have complied within the complete course of the experiments.

B. Carbamylation

Ovalbumin was purchased as a powder from Sigma-Aldrich Brasil Ltda EPP (Cotia – SP – Brazil). The carbamylation was performed after dilution of ovalbumin in a borax buffer (4 mg/mL) followed by the addition of potassium cyanate (0.5 M) and kept overnight at 40 °C [45], [46]. The final solution was submitted to NaCl (0.15M) and EDTA (10g/L) buffer dialysis adjusted to pH 5.0 for 24 hours to remove residual cyanate ions. Two blank ovalbumin solutions were prepared with (and without) potassium chloride (0.5 M) instead of potassium cyanate. The three final solutions were adjusted to pH 7,5 with HCl and to 1 mg/mL as quantified by Bradford methodology [47]. The phosphated ovalbumin (pOVA) was used only to control the electrophoretic running. The LAI tests were done parallelly with the purified ovalbumin (OVA) and the carbamylated ovalbumin (cOVA).

C. Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Electrophoresis Gell

We compared, side by side, the purified, the phosphated, and the carbamylated ovalbumin utilizing a Coomassie® Blue stained 6% resolving sodium-dodecyl sulfate acrylamide (6%) / bis-acrylamide (40%) electrophoresis gel (SDS-PAGE) performed with a Mini Protean Tetra Cell apparatus (Bio-Rad, CA, USA) [48]. Aliquots of 5 µL of each extract were applied to each lane. A 10-180 KDa molecular mass Thermo Scientific prestained protein ladder (PageRuler™, Thermo Fisher, MA, USA) was used to identify approximate molecular weights. After electrophoresis, the gel was stained with Thermo Fisher Coomassie® Blue to identify the protein molecular weight distribution.

D. Leucocyte Adherence Inhibition Test

Plasma samples were collected in heparinized collection tubes. The *ex vivo* challenge tests were performed as described previously [49]. Each patient's fresh plasma was divided into three parts and used in two simultaneous and paralleled *ex vivo* challenging tests with purified ovalbumin (OVA) and the carbamylated ovalbumin (cOVA), both controlled by the third 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 1 mg/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 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 unchallenged control sample; multiplied by 100 (%). To further calculate the Leukocyte Adherence Inhibition (LAI) the LAR was subtracted from 100 (%).

E. Statistical Analyses

Statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., San Diego, CA, USA). The data were reported as arithmetic means with 95% confidence intervals (CI) and standard deviations (SD). Differences in the means of matched samples were assessed by paired *t*-tests [50]. For all analyses, a p-value of less than 0.05 was considered significant. Two whiskers-and-box plot graphs were generated by the software.

III. Results

The Coomassie® Blue-stained gel similarly showed two groups of proteins at the purified ovalbumin lane, the carbamylated ovalbumin lane, and the phosphated ovalbumin lane. The lighter group observed at approximately 43 kDa corresponds to the monomeric alpha-helix. The heavier group with approximately 86 kDa corresponds to the aggregated beta-sheet. The carbamylation interfered with the binding of the dye to the proteins but did not affect significantly their molecular weight (see Fig. 1).

When comparing the paired LAI responses inside the whole tested population, there was no significant difference by the *t*-test between OVA and cOVA immunoreactivity. However, we observed two distinct groups according to the results. The first group was defined by the 16 individuals that presented a significant decrease in ovalbumin's immunoreactivity (as assumed by the LAI) after carbamylation (mean of differences = 35%; $p = 0.002$) as displayed in Fig. 2. The second group was defined by 17

individuals that presented a significant increase in ovalbumin's immunoreactivity (as assumed by the LAI) after carbamylation (mean of differences = 32%; p = 0.001) as displayed in Fig. 3. The main indicator that the carbamylation interfered randomly in the immunoreactivity to OVA was the fact that the previously paired challenge tests were considered "not paired" by the *t*-tests after carbamylation.

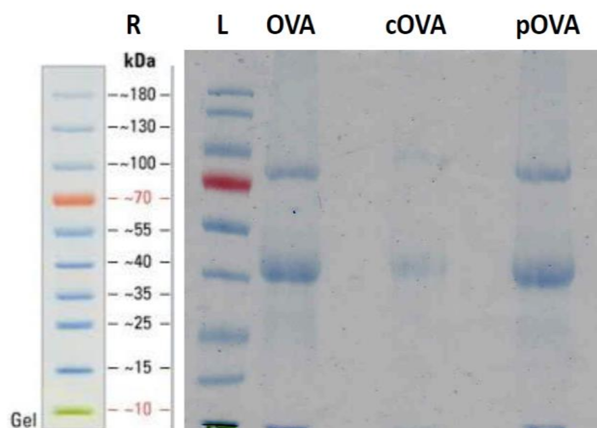

Fig. 1. Coomassie® Blue stained 6% resolving sodium-dodecyl sulfate (SDS)-polyacrylamide electrophoresis gel. R: Ruler; L: Ladder; OVA: ovalbumin; cOVA: carbamylated ovalbumin; pOVA: phosphorylated OVA.

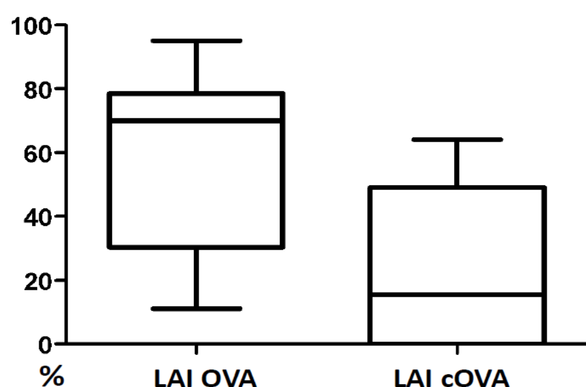

Fig. 2. Whiskers-and-box plot graph generated by GraphPad Prism software describing the paired *t*-test of the 16 individuals that presented a decrease on ovalbumin's immunoreactivity after carbamylation as monitored by Leukocyte Adherence Inhibition Test (mean of differences = 35%; p = 0.002).

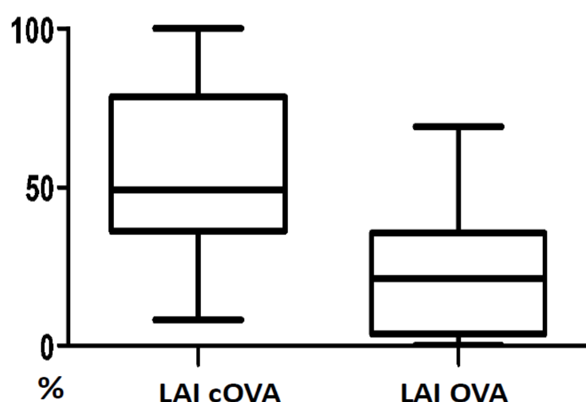

Fig. 3. Whiskers-and-box plot graph generated by GraphPad Prism software describing the paired *t*-test of the 17 individuals that presented an increase on ovalbumin's immunoreactivity after carbamylation as monitored by Leukocyte Adherence Inhibition Test (mean of differences = 32%; p = 0.001).

IV. Discussion

The design and the production of less allergenic food is a great concern not only for nutritional purposes but also for therapeutic interventions on food allergic people, mainly in this era characterized by the production of chemically, enzymatically, and/or genetically modified foods [51]. Usually, the concept of immunoreactivity employed by most food researchers with a nutritional background is exploited in its deleterious sense, referring to a reaginic immune reaction, usually mediated by IgE or IgG antibodies, associated with diseases, hypersensitivity or, at least, adverse reactions that can produce undesirable symptoms associated with the ingestion or even mere contact with specific food proteins [52]. But an immunologist must remember that the first and desirable immune reaction to food is immunotolerance. Tolerance to food is not the passive "absence" of immune reactions or unresponsiveness, but instead, is the resulting of an active and orchestrated interplay that establishes a chain of reconnaissance of specific nutritional proteins that the immune system identifies as beneficial to the body physiology. The major participants of this complex task are the tolerogenic Dendritic Cells, the regulatory T and B lymphocytes (Treg and Breg), the TGF-β, the IL-10, and the IgA [53]-[57]. The main determinant of the kind of immune response to be developed is the environment in which the allergens are collected by the Dendritic Cells before being presented to the naïve T Cells inside the lymph organs. According to the antigens' collecting environment, the Dendritic Cell expresses tolerogenic or costimulatory cytokines during the antigen presentation that drive the naïve T Cell to differentiate to a regulatory (tolerogenic) or a helper (inflammatory) phenotype [58]. When employing the LAI test to evaluate immunoreactivity we are not predicting the subsequent direction towards immunotolerance or hypersensitivity, but just the ability of the immune cells to recognize and respond to the specific antigen to which it is exposed. The inhibition of the leukocytes' glass adherence is just the final indicator of a non-specific release of cytokines. Therefore, this immunoassay is not designed to evaluate the utility of the LAI test to diagnosis allergy or to predict the patients' clinical response to the natural allergen or the carbamylated allergoid but to testify the first steps that drive the endotype differentiation. To predict the clinical response, it is necessary to employ information provided by others *in vivo, in vitro,* and *ex vivo* assays, such as the allergy cutaneous tests, the research of specific antibodies, the lymphocytes proliferation tests, or the basophils challenge tests, for instance. This experiment had the sole objective to explore the phenotype/endotype concept inside a Precision Medicine perspective. When evaluating the entire population, the statistical analysis found no significant difference in the immune response comparing the natural ovalbumin and the carbamylated allergoid. However, when the two different endotypes were defined, the statistical analyses revealed a significant difference characterizing the two trends of response. In conclusion, the objective of this work is to present a different perspective to evaluate the data, and instead of simply disqualify the mixed results, we propose to study more deeply the differences among the immune responses to extract additional information with the potential to improve and personalize the treatment of the therapeutic-

driven endotypes.

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