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Contribution of the Leukocyte Adherence Inhibition Test for the Evaluation of Immunoreactivity against Gluten Extracts in Non—IgE-Mediated / Non-Autoimmune Gluten-Related Disorders

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ABSTRACT

Background: **The Non-Celiac Gluten Sensitivity (NCGS) is a category inside the Gluten-Related Disorders (GRD) that groups the patients with unidentified mechanisms responsible for their symptoms.**

Objective: **To evaluate the opportunity of an *ex vivo* challenge immunoassay, the Leukocyte Adherence Inhibition Test (LAIT), to discriminate non—IgE-mediated gluten-specific immunoreactivity in patients with NCGS.**

Methods: ***Ex vivo*** **challenge tests performed with gluten latex extract, monitored by LAIT, were assayed in an asymptomatic control group of 30 individuals and a group of 52 patients with GRD not related to any identifiable immune mechanism (NCGS).**

Results: **The mean Leukocyte Adherence Inhibition (LAI) of the control group was 10.9%. The mean LAI of the NCGS patients' group was 54.9%. The non-parametric Wilcoxon-Mann-Whitney U test comparing the control group with the NCGS patient's group showed significance with a *p*-value < 0.00001.**

Conclusion: **The LAIT is an ex vivo challenge test able to discriminate gluten-sensitive and gluten-tolerant individuals, suggesting the participation of an immune Non—IgE-mediated hypersensitivity reaction in patients with the clinical diagnosis of NCGS.**

Keywords: Diet therapy, gluten, hypersensitivity, leukocyte adherence inhibition test.

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I. Introduction

The term "gluten" was originally employed to refer to the aqueous-insoluble gluey substance that remains after the flour is washed to remove the starch. Gluten is a complex mixture of hundreds of evolutionary-related cereal storage proteins whose main representants are the wheat alcohol-soluble gliadins and the wheat alcohol-insoluble glutenins [1]. Similar storage proteins exist in the rye (secalins), in barley (hordeins), and oats (avenins), also referred to as "glutens" [2]. Most gluten proteins are prolamins, as characterized by their high content of the nonessential amino acids proline and glutamine [3]. The characterization of gluten proteins is not an easy task, since their varietal components are dynamically evolving according to the crops [4], [5]. The Food Engineering searches, through crossbreeding, better varieties for cooking purposes, mainly for their aggregation and elastic properties as well the ability to form traps to secure the volatile carbon dioxide gas produced by microbial fermentation in breadmaking [6], [7]. The wheat flour with higher gluten content is semolina (21 – 32% of gluten), an intermediate milling stage of durum wheat, mainly used to cook pasta [8]. High-content gluten flours (12 – 14% of gluten) are produced from hard wheat and are usually used to cook bagels and pizza. Intermediate-content gluten flours (10-13% of gluten) are mainly used to cook bread. Low-content gluten flours (8 – 10% of gluten) are produced from soft wheat and are mainly used to make delicate cakes [9]. Multi-purpose wheat flours are a mixture of hard and soft wheat flours [10]. The gluten proteins are stable to heat, and their gastric/pancreatic partial proteolytic digestion produces intrinsically disordered peptides resistant to the intestinal peptidases, able to permeate the intestinal epithelium and to be secreted into the human milk [11]. Despite representing a

major nutritional source of amino acids for humans, the gluten proteins and their undigested peptides may trigger, in some individuals, hypersensitivity and autoimmune reactions able to produce debilitant diseases [12]. Gluten-Related Disorders (GRD) are a large set of heterogeneous diseases that have in common the dietary gluten as the triggering agent of an inflammatory reaction [13]. Despite intense research over these conditions, the physiopathology of these disorders is unsatisfactorily defined [14]. The medical literature is yet searching for an ideal nomenclature and classification criteria for them. To our clinical practice we arbitrarily classify the GRD in four groups according to the identified immune mechanisms associated with the gluten-induced inflammation: A) The autoimmune-related hypersensitivities; B) The IgE-mediated gluten (or wheat) allergy (the Gell and Coombs' type I hypersensitivity reaction); C) The non—IgE-mediated gluten (or wheat) allergy (either the Gell and Coombs' types II, III and/or IV hypersensitivity reactions); and D) The Non-Celiac Gluten Sensitivity (NCGS) [15]-[17]. The main autoimmune-related phenotypes associated with gluten hypersensitivity are celiac disease, dermatitis herpetiformis, and gluten ataxia [18]-[20]. The autoimmune-related hypersensitivities are characterized by distinct histopathology, the production of cross-reactive antigen-elicited autoimmune responses (either antibody-mediated or cell-mediated), and a Major Histocompatibility Complex (MHC) genetic-related predisposition [21], [22]. The IgE-mediated gluten hypersensitivities syndromes may be manifest by diverse phenotypes, with cutaneous, gastrointestinal, respiratory, and systemic symptoms such as atopic dermatitis, rhinitis, asthma, and anaphylaxis [23]-[26]. The IgE-mediated clinical syndromes are easily identifiable in clinical practice employing allergic skin tests and by the serum research of specific IgE antibodies against gluten-related allergens, actually performed by most clinical laboratories [27]. However, it is not uncommon to find patients who present immediate reactions to allergic skin tests, that do not present detectable serum-specific IgE against those allergens. It is most probable that these patients present cutaneous IgE-mediated reactions that were not detected by the common blood assays, usually limited to the serum compartment, performed in laboratories not prepared to assay the plasma [28]. A comparable phenomenon may occur in the recently described "Local Immune Response to Food Antigens", where the local production of specific IgE may induce intestinal-limited allergic inflammation [29]. As the gluten proteins are not the only proteins of the wheat, some IgE-mediated allergic reactions may involve non-gluten wheat proteins, some of them related to pollen [30], [31]. The non—IgE-mediated gluten immune hypersensitivities are indirectly suspected in the context of eosinophilic conditions; they usually are associated with other identifiable non—IgE-mediated food allergies; and are clinically diagnosed with skin patch tests and Oral Food Challenges (OFC) [32]. The leukocyte reactivity, as well the presence of antibodies against gluten compounds is indirect evidence of a type II Gell and Coombs' hypersensitivity reaction and may also be present in autoimmune conditions [33], [34]. The research of precipitins to dietary proteins is also indirect evidence of a type III Gell and Coombs' hypersensitivity reaction, and, as well, might be present in autoimmune conditions [35]. The GRD that do not present evidence to justify their classification into the other three categories are provisionally grouped under the inappropriate denomination "Non-Celiac Gluten Sensitivity" (NCGS), a set of undefined non—IgE-mediated/non-autoimmune syndromes manifested by intestinal and extra-intestinal symptoms [36]-[38]. The first example proposed for this heterogeneous group of disorders was described in patients suffering from gluten-dependent diarrhea, with no histological evidence compatible with celiac disease [39]. There is not a clear definition for NCGS, since the clinical criteria proposed for this syndrome may overlap other poorly defined clinical conditions such as the Irritable Bowel Syndrome (IBS), the non—IgE-mediated Gastrointestinal Food Allergy, the Food-Protein Induced Enteropathy Syndrome (FPIES), or any non—IgE-mediated immune gluten hypersensitivity producing systemic, gastrointestinal, respiratory or dermatologic disorders [40]-[43]. There is a considerable chance that most NCGS will eventually join the non—IgE-mediated immune hypersensitivities group, since a type II, III, and/or IV Gell and Coombs' mechanism may eventually be attributed to them [44].

Nowadays there is an overspread idealism around what is called "gluten intolerance", an indefinite generic term that has impregnated the nutritional practice around the world to distinguish the group of individuals that regularly ingest and tolerate gluten, from the group of individuals that present any adverse reaction related with dietary gluten [45]. It is not uncommon to find such patients, suffering from unspecified digestive disturbances that, empirically advised by their nutritionists, with little or any medical investigation, had proceeded empiric gluten-free diets, with improvement or resolution of their symptoms. These patients report that every time they try to ingest gluten, they present a resurgence of their symptoms, compelling them to undertake gluten-exclusion diets. The medical literature has also plenty of reports of similar disorders, describing diverse approaches and sometimes controversial interpretations over the possible physiopathology [46], [47]. The objective of this study is to employ the Leukocyte Inhibition Adherence Test (LAIT) as a tool to evaluate the immunoreactivity against gluten proteins in symptomatic NCGS patients, or, in other words: "gluten-intolerant" patients (under a well-succeeded gluten exclusion diet) that do not present any other evidence of autoimmune, IgE-mediated or non—IgE-mediated immune gluten hypersensitivity.

The Leukocyte Adherence Inhibition Test (LAIT) is an *ex vivo* challenge test designed to evaluate the inhibitory effect of specific antigens on the natural capacity of glass adherence of leukocytes [48]-[53]. When sensitized to specific antigens to which they are exposed, the live leukocytes release cytokines that interfere with glass adherence of nearby leukocytes, a phenomenon quantifiable with help of a concomitant assay done with unchallenged plasma [54]-[58]. This specific inhibition depends on the presence of specific antibodies, suggesting a type II Gell and Coombs antibody-dependent cellular-mediated immune response [59]-[62]. The use of the LAIT to evaluate the gluten immunoreactivity in GRD is not an original idea, since it has already been employed successfully in patients with celiac disease and dermatitis herpetiform [33].

II. Methods

A. Subjects

After receiving Institutional Review Board approval, from the Instituto Alergoimuno de Americana (Brazil), a group of 52 patients diagnosed as NCGS by clinical and laboratory criteria (13 male; 18 - 86 years old; mean age = 46 years, SD = 13.8 years) and a control group of 30 gluten-tolerant subjects (9 male; 22 - 67 years old; mean age = 46 years, SD = 13.8 years) were invited, with informed consent formularies, to voluntarily be submitted to allergy skin tests against gluten extract and provide blood samples to research specific gluten-specific IgE antibodies, gliadin-specific IgA antibodies, gliadin-specific IgG antibodies, and to perform *ex vivo* challenge tests, according to the principles of Helsinki and the International Committee of Medical Journals Editors requirements of privacy [63]. All patients of the symptomatic group had been referred by a gastroenterologist and had already been previously submitted to upper gastrointestinal endoscopic duodenal biopsy to discard the diagnosis of celiac disease. The asymptomatic control group was not submitted to this procedure. All NCGS patients presented an established relationship between gluten ingestion and gastrointestinal symptoms and were in a successful gluten-free diet, with several recurrences of symptoms after the ingestion of gluten. The research of gliadin-specific IgA and the research of gliadin-specific IgG were all below the detection limit of the laboratory method for both patients and controls. Patients and control-group individuals had non-detectable serum-specific IgE and non-reactive skin tests against gluten extracts and at least more than 30 other diverse respiratory and food allergens [64]. The study was descriptive, retrospective, 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 been complied with, within the complete course of the experiments.

B. Gluten Extraction

Multi-purpose wheat flour was bought from a local supplier. In a beaker, 20 g of multi-purpose wheat flour was added to 100 mL of distilled water. The sample was stirred for 30 minutes and centrifuged at 4,500 rpm for 10 minutes. The process was repeated four times to wash and remove the starch. After washing the precipitate was resuspended in 50% ethyl alcohol and stirred for 30 minutes. Then the sample was filtered and centrifuged at 4,500 rpm for 10 minutes. The protein quantification of the allergen extracts was done according to Bradford's protein-dye binding methodology [65]. The gluten extract was diluted to an estimated protein concentration of 1 mg/mL and stored at 4 °C. All relevant and mandatory laboratory health and safety measures have been complied with in the complete course of the experiments.

C. Leukocyte Adherence Inhibition Test

Plasma samples were collected in heparinized collection tubes. The *ex vivo* challenge tests were performed as described previously [66]. Shortly, each donor's fresh plasma was divided into two parts and used in paralleled *ex vivo* challenging tests with gluten extract 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 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 (%). From blood collection to the immunoassay final report, it takes 4 hours.

D. Graphic Presentation of Data and Statistics

A column graph was plotted with the mean LAIT results of each group (Fig. 1). The data of the patients' groups were compared with the control group by the non-parametric Wilcoxon-Mann-Whitney U test [67], [68].

III. Results

The mean LAI of the control group was 10.9% (range = 0% – 40%; SD = 13.5%). The mean LAI of the complete patients' group was 54.9% (range = 0% – 93%; SD = 27.23%). The non-parametric Wilcoxon-Mann-Whitney U test comparing the control group with the NCGS patient's group showed significance with a p-value < 0.00001.

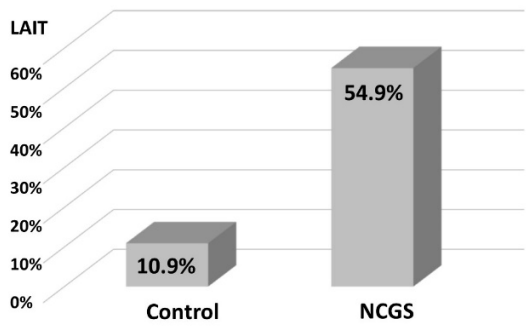

Fig. 1. Column comparison chart with the average Leukocyte Inhibition (%) of the *ex vivo* challenge tests performed with gluten extract, monitored by Leukocyte Adherence Inhibition Tests, comparing the control group with the non-celiac gluten-sensitive patients' group (NCGS).

IV. Discussion

In the diagnostic setting of allergic diseases, most physicians usually are satisfied when find an IgE-mediated hypersensitivity and stop the clinical investigation. However, the patient may also be under the influence of concomitant uncovered non—IgE-mediated hypersensitivities. The three distinct non—IgE-mediated categories of immune mechanisms characterized by Gell and Coombs are rather a broader vision of immune interactions, described according to the main participants of the hypersensitivity reactions. However, the Gell and Coombs' categories are a simplistic description based on the knowledge acquired until the sixties, ignoring the participation of the cytokines and their membrane receptors, the key components of any immune reaction. Among the main advances that allowed a better comprehension of these phenomena are the *ex vivo* allergen-specific immunocyte stimulations, able to define the cytokine profile of allergic T cells clones and characterize different *ex vivo* profiles or phenotypes [69]-[72]. The LAIT is not a profiling immunoassay able to define specific phenotypes but is proposed to serve as a feasible clinical tool to intermediate a link between the expensive medical research and the unfortunate allergic patients in the triage diagnosis of allergen-specific non—IgE-mediated immunoreactivity.

The participation of food allergies and/or the food adverse reactions in gastrointestinal diseases have been historically a controversial issue, as well a matter of debate and dispute [73], [74]. In adult patients with food-induced gastrointestinal symptoms, the OFC usually does not correlate well with the research of specific IgE, suggesting the presence of different mechanisms of hypersensitivity [75]. Among the described participants of the allergic march, food allergy is the most neglected condition [76]. The explanation for this is that the other participants (eczema, allergic rhinitis, and asthma) are clinically defined groupings of compartmentalized inflammatory signs and symptoms diagnosed by anamnesis and physical examination, while food allergy is an underlying causal systemic disease that may produce, besides the systemic and gastrointestinal disorders, also the respiratory and cutaneous signs and symptoms attributed to eczema, allergic rhinitis, and asthma [77]. The inability to identify a causal relationship between an allergen trigger and an allergic reaction leads the clinician to tag the patient with the "idiopathic" exclusion diagnosis [78]. The "idiopathic" nickname for any hypersensitivity reaction, also described by the concept of "intrinsic" allergy, is suggested by the existence of two phenotypes of patients with absolutely the same clinical presentation and different IgE profiles [79]-[81]. The suggestion by the LAIT of a non—IgE-mediated immune reaction originating these "intrinsic" hypersensitivities, steps up the comprehension of these phenomena to a novel level, conceptualizing more effective diagnostic tools and therapeutic approaches [82], [83]. With the advance of knowledge, the improvement of old diagnostic assays, and the development of new tools, medical science is narrowing the "idiopathic" diagnosis, describing the physiopathology of poorly understood diseases, to better help the suffering patients that search for medical guidance [84]. The suspicion of gluten hypersensitivity is capital for the indication of diagnostic Oral Food Challenges and the prescription of immune therapeutic interventions such as gluten-free diets that, providing limitation of the access to feeding antigens, restrains the plasmablast responses, improving the immunologic status and the clinical symptoms [85].

The significant difference between the mean LAI of the control group and the patients' groups demonstrated that the *ex vivo* challenge test performed with the gluten extract, monitored by the LAIT, can differentiate the specific immunoreactivity between the control group and the NCGS group. The largest LAI found in the control group was 40%, which may be attributed to an asymptomatic sensitization. Most *ex vivo* challenge tests from the control group resulted in the LAI = 0%. The finding of immunoreactivity against an allergen as demonstrated by the LAIT does not confirm the diagnosis of a hypersensitivity allergic disease. The relationship between the immunoreactivity demonstrated by the LAIT and the effective participation of the allergen in the appearance of symptoms may only be determined by a careful *in vivo* challenge test initiated with a successful exclusion diet and a controlled re-introduction of the allergen through *an in vivo* challenge test. The *in vivo* challenge tests are empirical, sometimes risky, laborious, time-wasting, and depend on a previous successful exclusion diet to allow the resurgence of the allergic symptoms. The possibility of a triage done by *ex vivo* challenge tests with the purpose of pre-selecting a group of antigens to proceed with the exclusion diet and the further *in vivo* oral challenges tests may save time and effort. The inhibition of the leukocytes' glass adherence is a clue about a specific immunoreactivity against the challenged antigen. The LAIT indicates the unspecific release of cytokines after the encounter with a specific antigen and proved to be an easy, quick, and inexpensive *ex vivo* immunoassay with the potential to predict individual immunoreactivity against gluten allergens in real-world patients with non—IgE-mediated hypersensitivity [86]. The clinical investigation nowadays is yet far from the complex immunoassays designed to identify and quantify the cytokines liberated after cellular *ex vivo* challenges performed with the suspected allergens, however, the LAIT acts as a bench-to-bed tool, easily performed at any laboratory facility equipped with basic equipment and trained staff to perform the immunoassay. Although the data generated by this exploratory trial appear to be consistent, a confirmation of these findings by more broad and specific studies will be highly welcomed.

Conflict of Interest

Authors declare that they do not have any conflict of interest.

Abbreviations

GRD: Gluten-Related Disorders
LA: Leukocyte Adherence
LAR: Leukocyte Adherence Ratio
LAI: Leukocyte Adherence Inhibition
LAIT: Leukocyte Adherence Inhibition Test
NCGS: Non-Celiac Gluten Sensitivity

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