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Leukocyte Adherence Inhibition Test to the assessment of Immunoreactivity Against Cow's Milk Proteins in Non—IgE-Mediated Gastrointestinal Food Allergy

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ABSTRACT

Background: **The non—IgE-mediated food allergy syndromes are a group of conditions diagnosed mostly by clinical criteria and Oral Food Challenge tests (OFC).**

Objective: **To evaluate the feasibility of the Leukocyte Adherence Inhibition Test (LAIT) to discriminate antigen-specific immunoreactivity in a group of patients with non—IgE-mediated cow's milk allergy diagnosed by OFC and clinical criteria.**

Methods: ***Ex vivo*** **challenge tests performed with cow's milk proteins extracts were monitored by LAIT in two groups: A) the active group consisting of 38 patients presenting non—IgE-mediated gastrointestinal lactose-free cow's milk hypersensitivity; B) the control group consisting of 29 cow's milk tolerant individuals**

Results: **The mean Leukocyte Adherence Inhibition (LAI) of the control group was 5.48%, and the mean LAI of the active group was 54.18%. The non-parametric Wilcoxon-Mann-Whitney U test showed $p < \alpha = 0.05$.**

Conclusion: **The Leukocyte Adherence Inhibition Test is an *ex vivo* immunoassay able to predict individual non—IgE-mediated immunoreactivity against cow's milk allergens and to act as a triage test to select food allergens to be prescribed in exclusion diets to perform OFC in patients with suspected non—IgE-mediated food allergies.**

Keywords: Adult, allergy, antigen, food, cellular immunity, enterocolitis, hypersensitivity, immunoassay, leukocyte adherence inhibition test, milk.

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I. Introduction

The non—IgE-mediated hypersensitivities syndromes are a large group of diverse clinical conditions yet poorly understood. The four basic divisions proposed by Gell and Coombs in the 1960s is a descriptive classification, based on the recognized participating elements of each group of reactions, which does not explore the complex interrelationships among the different immunological segments, as yet not described, such as the cytokines [1]. The link between the leukocyte's functional properties and the non—IgE-mediated hypersensitivities of clinical syndromes is an active field of investigation. The concept of "food allergen-activated neutrophils" was recently demonstrated by studies on nuclear and mitochondrial DNA release and associated with the non—IgE-mediated food allergy [2]. Several studies suggest that the methylated DNA possesses immunosuppressive properties activating the regulatory T-cells (Treg), while the nonmethylated DNA induces the production of proinflammatory cytokines [3]-[5]. The so-described "Non–IgE-mediated gastrointestinal food-induced allergic disorders", also called "Non–IgE-mediated gastrointestinal food allergy", represent a wide range of complex and clinically diversified entities whose immune mechanisms were not yet completely elucidated [6]-[8]. Among these entities, Food Protein-Induced Enterocolitis Syndrome (FPIES) is clinically well-characterized in children but poorly defined in adults [9], [10]. The increased level of IL-8 after a positive OFC performed with cow's milk proteins suggests that the neutrophils are involved in the pathophysiology of FPIES [11].

In a clear attempt to avoid confusion with the pediatric FPIES, Tan and Smith defined, in 2014, the FPIES-similar adult-onset entity called "Non—IgE-mediated gastrointestinal food hypersensitivity syndrome in adults" (we will employ the abbreviation "NGIFA" to refer to this syndrome) [12]. In the lack of a validated biomarker, these authors defined this syndrome with a set of clinical

characteristics, as quoted: "A) Acute severe abdominal pain, repetitive vomiting, and/or diarrhea; B) Reproducible on ingestion of specific foods on at least 2 separate occasions; C) Symptoms isolated to the gastrointestinal tract with the absence of cutaneous, respiratory, or cardiovascular signs or symptoms of allergy; D) Symptom resolution with avoidance of incriminated food; E) Absence of food-specific IgE on skin prick tests and/or serology; F) No other cause for symptoms identified; G) Age greater than 18 years". Despite most patients of the Tan and Smith series being sensitive to seafood, it came to our attention that these clinical features were equivalent to the symptoms presented in two other distinct conditions: a clinically defined entity, the Irritable Bowel Syndrome, as well a genetic enzymatic deficiency, the Adult-onset Lactose Intolerance [13], [14]. These gastrointestinal food hypersensitivity symptoms are rarely produced by seafood ingestion among our hypersensitive patients, which present mostly urticaria or respiratory symptoms associated with these allergens. However, these symptoms are more commonly reported by our patients after the ingestion of lactose-free cow's milk. We attend a non-coastal area located in the interior of a large country, where seafood consumption is not a habit, with big cattle pasture areas where the milk production is at large. The group of patients studied here meets the Tan and Smith criteria after an Oral Food Challenge test (OFC) performed with lactose-free cow's milk [15]. Until the dissemination of the production of lactose-free milk derivatives, it was somehow difficult to differentiate the pure lactose intolerance from the pure Non–IgE-mediated cow's milk allergy, since the two conditions may coexist [16]. Nowadays it is easy to perform an OFC with a lactose-free milk derivative and observe the symptoms. There is a natural difficulty to perform Doble-Blind Placebo-Controlled OFC with cow's milk since the milk flavor and taste are too characteristic to prevent the identification of the full glass ingested by the adult patient, so most OFC done with cow's milk in real-world medical facilities are open [17]. The gold-standard clinical procedure to confirm the diagnosis of food allergy is yet the in vivo OFC, a clinical procedure that does not reveal any immune or non-immune mechanism of hypersensitivity [18]. Nowadays there is excessive confidence in the research of specific IgE and allergic-skin tests to diagnose allergies [19]. These tests, mainly when negatives, are not confirmatory and overlook the diagnosis of non—IgE-mediated hypersensitivities that may be clarified by a thorough clinical history and the well-guided OFC [20]. Among monosensitized allergic patients, the OFC is a simple procedure, performed only with the suspected food allergen. However, in polysensitized patients, the OFC may be a complex procedure that depends on a previous empiric elimination diet to effectively clear the symptoms before the reintroduction of the suspected allergen. The feasibility of an *ex vivo* blood pre-test, that may detect immunoreactivity to specific allergens, as a tool to select specific foods to proceed with elimination diets before the OFC, is a desirable possibility that may save valuable time and resources, turning less empirical the prescription of the pre-OFC exclusion diet. In the search for an immune biomarker for this particular condition, we realized that cow's milk is the main culprit for the pediatric FIPES, which main immune characteristic is the active involvement of leukocytes, as noticed in stool samples examined after Oral Food Challenges (OFC) [21]. Recently, the Leukocyte Adherence Inhibition Test (LAIT) has been exploratively evaluated, as a potential tool to demonstrate non—IgE-mediated hypersensitivity against food allergens, such as the proteins associated with latex and gluten [22], [23]. To evaluate the ability of LAIT to demonstrate immunoreactivity against cow's milk proteins, we compared its performance between the heparinized plasma of outpatients with lactose-free cow's milk-induced NGIFA (according to the OFC and the Tan and Smith's criteria) and the heparinized plasma of cow's milk-tolerant control individuals.

II. Methods

A. Subjects

After receiving Institutional Review Board approval, from the Instituto Alergoimuno de Americana (Brazil), a group of 38 outpatients (13 male; 25-76 years old; mean age = 47 years, SD =16 years) clinically selected by Tan and Smith's criteria associated to a positive lactose-free cow's milk-induced OFC; and a control group of 29 cow's milk-tolerant voluntary donors (5 male; 19-78 years-old; mean age = 49 years, SD = 16 years) were invited, with informed consent formularies, to be submitted to skin-allergic tests and voluntarily provide blood samples to perform *ex vivo* challenge tests, according to the principles of Helsinki and the International Committee of Medical Journals requirements of privacy [24]-[26]. All patients and control-group individuals had non-detectable specific IgE and non-reactive skin tests to cow's milk proteins. 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. Cow's milk proteins extraction

In a beaker, 50 g of skim powdered cow's milk was added to 25 mL of the diluent solution (NaCl 10 g; KH_2PO_4 0,72 g; Na_3PO_4 2,86 g; methylparaben 1 g; propylparaben 0,5 g; glycerin 400 mL; H_2O 600 mL). The protein quantification of the allergen extracts was done according to Bradford's protein-dye binding methodology [65]. The protein extract was diluted, with the same diluent, to the 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 [27]. Shortly, each donor's fresh plasma was divided into two parts and used in paralleled *ex vivo* challenging tests with cow's milk 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 skimmed lactose-free cow's milk 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 the unchallenged control sample; multiplied by 100 (%). To further calculate the Leukocyte Adherence Inhibition (LAI) the LAR was subtracted from 100 (%).

D. Graphic Presentation of Data and Statistics

Two distribution tables were constructed to allow an overview of the contribution of the LAIT inside the context of each NGIFA patient and each control subject (tables 1 and 2). Based on these tables, the accuracy of LAIT (false positives and false negatives) was calculated assuming for reference the diagnostic criteria proposed for Tan and Smith associated with the positive OFC. The data of the two independent groups were compared by the non-parametric Wilcoxon-Mann-Whitney U test [28], [29].

III. Results

There was a significant difference between the mean LAI of the two groups (see Fig. 1). The mean LAI of the control group was 5.48%, (0 – 31%; SD = 10.27%) while the mean LAI of the NGIFA group was 54.18% (0 – 95%; SD = 22.40%). The non-parametric Wilcoxon-Mann-Whitney U test showed $p < \alpha = 0.05$ meaning that it is a statistically significant difference between the groups of values ($Z = -6.6811$, which is not in the 95% region of acceptance: [- 1.96 : 1.96]; $U = 32$ which is not in the 95% region of acceptance: [398.8928 : 703.1072]). From the control group, 72,4% (21/29) presented LAI = 0,0% and the greater LAI was = 33%. From the NGIFA group, 2,6% (1/38) presented LAI = 0,0% and 7,9% (3/38) presented LAI < 33%. Considering only these data, if we elect an LAI = 0,0% as the reference value, the false-negative rate of the test to predict an immune response against cow's milk proteins is 2,6% and the false-positive rate is 27,6%. However, if we elect an LAI > 33% as the cut-off reference value to predict an immune response against cow's milk proteins, the false-positive rate is 0,0% and the false-negative is 7,9%.

TABLE I: TABULATION OF SEX, AGE, AND RESULTS OF THE LEUKOCYTE ADHERENCE INHIBITION TESTS PERFORMED WITH COW'S MILK IN 38 PATIENTS WITH NON—IGE-MEDIATED COW'S MILK GASTROINTESTINAL FOOD HYPERSENSITIVITY SYNDROME

Patients	Sex	Age	LAIT	Patients	Sex	Age	LAIT
ADS	F	30	6	MIC	F	25	82
ADZ	F	42	59	MUR	M	30	75
ALP	M	70	59	NAC	F	26	79
CLA	M	35	49	NAR	F	33	53
CLC	F	64	47	NAZ	M	70	43
DEO	F	75	21	RIC	M	58	79
ELS	F	43	47	RIM	M	28	61
GEN	M	40	36	ROB	M	64	45
GLA	F	39	95	ROF	F	51	44
ISA	F	74	82	ROSA	F	62	95
JOW	M	53	77	ROSE	F	67	40
JOZ	M	76	58	SAM	F	32	38
LAR	F	55	56	SAN	F	40	30
LUI	M	34	53	SEV	M	31	45
MAD	F	57	51	TAB	F	29	42
MAE	F	58	66	THI	M	34	86
MAF	F	61	41	VAC	F	26	36
MAI	F	47	35	VAF	F	38	0
MAK	F	43	65	VIL	F	62	83

Sex (M: male; F: female); age (years), and results of the Leukocyte Adherence Inhibition Test (LAIT - %)

TABLE III: TABULATION OF SEX, AGE, AND RESULTS OF THE LEUKOCYTE ADHERENCE INHIBITION TESTS) PERFORMED WITH COW'S MILK IN 29 CONTROL INDIVIDUALS, TOLERANT TO COW'S MILK

Control	Sex	Age	LAIT	Patients	Sex	Age	LAIT
APS	F	49	0	KRC	F	43	0
BNC	F	25	0	LFS	F	70	0
CIS	F	71	0	LOU	F	71	0
DEB	F	38	0	LUA	F	28	11
DED	F	58	17	LUC	F	34	0
EDC	F	50	0	MAG	F	70	0
ELC	F	48	0	MAH	F	42	0
ELU	M	38	0	MAJ	F	58	0
GFG	F	63	0	NA	F	42	0
IVA	F	64	13	NAB	F	28	0
JAR	F	58	0	NAD	F	19	0
JIC	M	55	26	PCK	F	31	6
JOS	M	57	22	VIV	F	33	31
JSF	F	65	33	WAL	M	78	0
JUB	M	43	0				

Sex (M: male; F: female); age (years), and results of the Leukocyte Adherence Inhibition Test (LAIT - %)

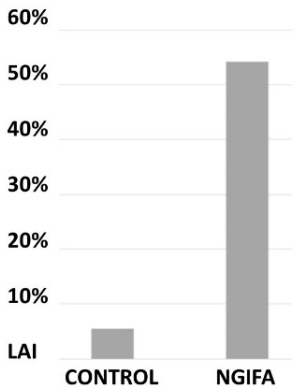

Fig. 1. Graphic distribution of the means of the Leukocyte Adherence Inhibition (LAI %) performed by *ex vivo* challenge tests with cow's milk proteins in two groups of adult subjects: a group of 38 patients with non—IgE-mediated cow's milk gastrointestinal food hypersensitivity syndrome (NGIFA); and a control group of 29 cow's milk tolerant individuals.

IV. Discussion

The cow's milk proteins may produce a wide range of IgE-mediated and non-IgE-mediated hypersensitivity syndromes characterized by distinct antibodies profiles and diversified lymphoproliferative responses [30]. The cellular participation in non-IgE mediated hypersensitivity against antigenic proteins had already been explored by several *ex vivo* challenge methods. The first *ex vivo* challenging method was described by Boyden in 1961 who designed a migratory chamber to study the effect of the mixture of antigens and antibodies on the migration of polymorphonuclear leukocytes [31]. Later, independent researchers, demonstrated by different techniques, a depressed neutrophil's chemotaxis in infants with cow's milk allergy [32], [33]. The LAIT was designed by Halliday, in 1972, to evaluate the inhibitory effect of specific antigens on the glass adherence of leukocytes [34], [35]. Used initially to study cellular reactivity against tumoral cells, the Leukocyte Adherence Inhibition Test (LAIT) was further employed to describe cellular immunoreactivity against different antigens, such as house dust mite, bacterial antigens, fungal antigens, and food proteins [36]-[39]. The natural capacity of live leukocytes to adhere to glass can be easily observed with an optical microscope and a glass surface hemocytometer chamber [40]. When functionally activated by specific antigens, leukocytes release paracrine soluble factors that interfere with glass adherence of nearby leukocytes, a nonspecific phenomenon, acting just as the final indicator of the specific leukocyte immunoreactivity that can be quantified with a concomitant assay done with unchallenged plasma [41]-[45]. Besides the cellular involvement, the adherence inhibition phenomena also may require the participation of specific IgG antibodies, suggesting a type II Gell and Coombs antibody-dependent cellular-mediated immune response [46]-[48]. The LAIT or the quantification of allergen-specific IgG antibodies against food allergens are not clinically recognized diagnostic assays, however, they may be proposed as triage tests to reduce the empiricism of the prescribed exclusion diets before initiating a diagnostic OFC [49]-[53].

The definition of the gastrointestinal syndromes produced by non—IgE-mediated hypersensitivity is yet a great challenge to the clinicians, due to the lack of immunoassays able to demonstrate their mechanisms. Besides the diseases that produce visible histological signs, such as celiac disease or eosinophilic inflammations, the diagnosis of the functional disturbances is yet established with oral challenges that do not demonstrate the responsible immune mechanisms. The suspicion of the immune participation is based, as a rule, on unspecific findings such as the presence of leukocytes, antibodies, or the increase of cytokines that do not elucidate the complete pathway between the allergen ingestion and the production of the symptoms. Food allergens may elicit the production of antibodies, but the real question to answer is when and in what circumstances these antibodies are responsible for the disease, responsible for the tolerance, or competing in both directions [54]. While the respective immune mechanisms are not fully elucidated, in clinical practice, the in vivo OFC remains the standard diagnostic procedure to "confirm" a food "allergy", although what the OFC really demonstrates is just a specific "adverse reaction". The rationale behind the additional use of any allergen-specific *ex vivo* challenge test, always associated with the OFC, is to concomitantly demonstrate the presence of an immune mechanism that may be held responsible for the diagnosis of an (at least) classifiable immune hypersensitivity (or "allergy") to a specific allergen. The demonstration of specific immune responses is a compelling reason to consider implementing a desensitization strategy [55], [56]. There is no rationale to consider desensitization strategies to treat non-immune intolerances. There is a tendency nowadays to employ *ex vivo* challenging methods stimulating immune cells with extracts of allergens to evaluate their response to specific antigens as well to allergen immunotherapy [57]. The LAIT is an allergen-specific *ex vivo* challenge test that suggests the involvement of a type II Gell and Coombs hypersensitivity reaction [58]. The LAIT demonstrates the inhibition of the leukocytes' glass adherence due to a non-specific release of cytokines after the specific recognition of the challenged antigen [59]. It had been employed in the past to demonstrate non—IgE-mediated hypersensitivity against diversified antigens, and in our experiments, the LAIT results were able to predict the clinical response of the food challenge against milk proteins in patients with non—IgE-mediated gastrointestinal food hypersensitivity syndrome, according to the Tan and Smith's criteria. Now a day, the performance of *ex vivo* challenge tests may be accomplished by the direct research of cytokines releases, such as the interferons and interleukins, instead of their effects on adherence or migration [60]. However, the LAIT does the same trick at a much lower cost. The LAIT is not designed to demonstrate a specific immune pathway, able to help scientific research; however, it demonstrates that there is an immune pathway, able to help a clinical investigation. Our results demonstrated that the LAIT is potentially a useful tool to be employed as a triage test to predict *ex vivo* immunoreactivity against specific cow's milk allergens to guide the choice of the food allergens to participate in the exclusion diets of candidates to OFC among patients suspected of non—IgE-mediated food hypersensitivities. Further studies are necessary to also consider its use in the evaluation of candidates for allergen immunotherapy, as well their response to treatment.

Conflict of Interest

The authors declare that they do not have any conflict of interest.

Abbreviations

LA: Leukocyte Adherence
LAR: Leukocyte Adherence Ratio
LAI: Leukocyte Adherence Inhibition
LAIT: Leukocyte Adherence Inhibition Test
OFC: Oral Food Challenge test
NGIFA: Non-IgE-mediated gastrointestinal food hypersensitivity syndrome in adults

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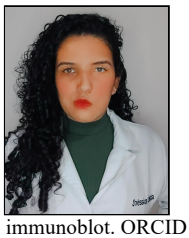

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