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Contribution of the Leukocyte Adherence Inhibition Test to the Diagnosis of Non–IgE-mediated Immunoreactivity against *Candida albicans* in Patients with Atopic Dermatitis

Celso Eduardo Olivier [a*], Daiana Guedes Pinto [a], Ana Paula Monezzi Teixeira [a], Jhéssica Letícia Santos Santana [a], Raquel Acácia Pereira Gonçalves Santos [a], Regiane Patussi Santos Lima [b] and Everton Salgado Monteiro [c]

[a] Instituto Alergoimuno de Americana, Brazil.
[b] Lavoisier's Laboratories, São Paulo, Brazil.
[c] Department of Allergy and Immunopathology, Faculty of Medicine, São Paulo University, Brazil.

Authors' contributions

The authors carried out this work in collaboration. The author CEO designed the study, wrote the protocol and the manuscript's first draft and managed the literature searches. Author ESM extracted the studied allergens. Authors DGP, APMT, JLSS and RPSL performed laboratory research. Author RAPGS performed cutaneous tests. All authors read and approved the final manuscript.

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**Corresponding author: E-mail: celso@alergoimuno.med.br;*

ABSTRACT

Aims: To evaluate the potential of the Leukocyte Adherence Inhibition Test (LAIT) to discriminate Non–IgE-mediated immunoreactivity against *Candida albicans* in Atopic Dermatitis () patients with clinical suspicion of hypersensitivity reactions to fungal allergens.
Study Design: We retrospectively examined the medical charts of 100 patients diagnosed with with clinical suspicion of Non–IgE-mediated fungal hypersensitivity who were investigated with an ex vivo challenge monitored by LAIT against an extract of *Candida albicans*.
Place and Duration of Study: Instituto Alergoimuno de Americana – São Paulo – Brazil – between January 2018 and October 2023.
Methodology: The percentage of Leukocyte Adherence Inhibition (LAI) promoted by the ex vivo challenges with *C. albicans* extract was distributed in ranges through a cascade distribution chart to outline the variability of the results.
Results: The mean LAI was 41.5%; SD 29.7%, ranging from 0% to 100%; mode = 0% (appeared 17 times). A wide distribution of LAI results suggested that some patients had immunoreactivity against the *Candida albicans* allergens while tolerant ones did not.
Conclusion: Our preliminary results support that the LAIT performed with *Candida albicans* may differentiate diverse degrees of ex vivo immunoreactivity against this airborne allergen in allergic patients.

Keywords: Allergy; Candida albicans; atopic dermatitis; diagnosis; hypersensitivity; leukocyte adherence inhibition test; non–IgE-mediated immunoreactivity.

1. INTRODUCTION

Candida albicans and *Saccharomyces cerevisiae* are Ascomycota yeasts classified at the Subphylum Saccharomycotina (formerly known as Hemiascomycota), belonging to the Class Saccharomycetes and the Order Saccharomycetales [1-3]. These yeasts exhibit remarkably adaptable heterotrophic metabolisms, enabling them to adapt to diverse ecosystems such as the human gastrointestinal, genital, or cutaneous microbiome (or mycobiome) [4]. *C. albicans* has evolved as a human commensal through millennia, diversifying into several clades geographically distributed worldwide [5]. *C. albicans* has a unique reproduction cycle with haploid, diploid, and tetraploid forms: a highly dynamic genome with extensive karyotypic variations that allow a singular plasticity [6]. This genotypic plasticity provides *C. albicans* the ability to switch between two distinct morphological states, evolving from an asymptomatic commensal oval yeast to an invasive hyphal-growth pathogen [7]. When the yeast form of *C. albicans* adheres to epithelial cells and keratinocytes, it can induce three kinds of signaling mechanisms: A) the nuclear factor-kappaB (NF-κB) pathway; B) the phosphatidylinositol-4,5-bisphosphate 3-kinase (Pi3K) pathway; C) the mitogen-activated protein kinase (MAPK) pathways {ERK1/2 (Extracellular signal-Regulated protein Kinase) and p38 c-Jun N-terminal Kinase (JNK)} [8-10]. The adherence of *C. albicans* to the host's cells activates the respective signaling pathways but does not necessarily induce the production of proinflammatory cytokines. Cellular adhesion is a potent inducer of hypha formation, which is accompanied by the expression of hypha-associated proteins, such as Hwp1 (Hyphal wall protein 1) and Als3 (Agglutinin-like sequence 3) [11-15]. Als3 is both an invasion promoter and an adhesin, promoting the endocytosis of *C. albicans* into epithelial cells along with heat shock proteins [16-19].

The secretion of Candidalysin and the differentiation of hypha are critical for host cell damage and immune activation, characterized by the recruitment of innate immune cells such as macrophages, neutrophils, and innate Type 17 cells [20,21]. Fungi express at their cell membranes Pathogen-Associated Molecular Patterns (PAMPs), which are recognized by host innate Pattern Recognition Receptors (PRRs) [22]. The main innate PAMP constituents of the *C. albicans* cell wall are mannan (and mannoproteins), β-glucan, and chitin. It is supposed that mannan preferentially stimulates a tolerogenic immune response through the mannan receptors since it covers β-glucan and chitin, which, only after exposition, stimulate potent innate proinflammatory responses through their receptors, such as the Dectin-1 and chitin receptors on the surface of innate immune cells [23]. Neutrophils are the primary mechanism of defense against *C. albicans* through phagocytosis, degranulation, the production of

Reactive Oxygen Species (R.O.S.), and the formation of Neutrophil Extracellular Traps (NETs) [24]. Neutrophils can phagocytize the yeast form of *C. albicans*. However, the hyphae are too large to be engulfed, so forming NETs is the better alternative to eliminate them [25]. Co-infections of *C. albicans* and *Staphylococcus aureus* are particularly prone to increase immune dysregulation, as they generate metabolic changes that increase virulence, cell wall remodeling, and hyphae morphogenesis in *C. albicans* also enhancing toxin production by *S. aureus* [26]. It is also reasonable to suppose that the immune aggression provided by the host during interaction with fungi and bacteria may increase *C. albicans* virulence. The mobilization of the innate immune arm activates the adaptive immune arm through antigen presentation, producing specific antibodies [27]. The saprophyte-to-pathogen transition of *C. albicans* relies mainly on the immune mechanisms responsible for controlling fungal proliferation, such as immune suppression and hypersensitivity [28]. Dendritic Cells critically balance inflammation and tolerance to yeasts, pivotally orchestrating the immune reaction towards commensalism or pathogenicity. Inflammatory D.C.s initiated Th17/Th2 responses to *C. albicans*. In contrast, tolerogenic D.C.s activate Th1/T regulatory cell (Treg) differentiation, exploiting the tolerogenic activity of the fungus [29]. The imbalance towards Th17/Th2 polarization drives the production of IgE-mediated hypersensitivity reactions against *C. albicans* allergens associated with impairment of cell-mediated Immunity [30,31].

Several allergens of *C. albicans* had significant levels of homology and strongly cross-reacted with the homologous constituents of *S. cerevisiae* [32,33]. However, due to its high plasticity, the allergen composition of *C. albicans* varies significantly [34]. SDS-PAGE immunoblotting revealed IgE, IgA, and IgG antibodies mainly directed against a *C. albicans* 46 kDa mannan-linked protein and 15 other IgE-bounding antigenic bands [35]. ELISA showed complete reciprocal cross-inhibition of the binding of specific IgE against mannoproteins of *Pityrosporum ovale* and *C. albicans* in patients with Atopic DermatitisAD [36]. Finnish researchers found a significant correlation between intestinal colonization and IgE sensitization (positive skin tests and serum-specific IgE) against *C. albicans* in patients with AD Most patients also had IgA and IgG antibodies against *C. albicans* mannan or their proteins [37]. Japanese researchers reported that antifungal drugs markedly improved the AD manifestations of patients presenting IgE-sensitization against *C. albicans* [38]. It is supposed that the immune aggression provided by the host during interaction with fungi and bacteria may be a factor in increasing *C. albicans* virulence [39].

In search of a propaedeutic tool to include the innate immune arm activity when evaluating patients suffering from pathogenic *C. albicans*, we employ the Leukocyte Adherence Inhibition Test (LAIT) performed on our installations. The LAIT is a simple and quick *ex vivo* laboratory procedure made with viable leukocytes, demonstrating immunoreactivity against fungal allergens such as *C. albicans*, edible yeasts, and mold allergens [40-44].

To evaluate the potential of the LAIT to reproduce Non–IgE-mediated immunoreactivity against *C. albicans*, we retrospectively examined the medical charts of patients investigated with an *ex vivo* challenge monitored by LAIT against a *C. albicans* extract. These patients, diagnosed with AD, had clinical suspicion of allergic reactions to fungal allergens, non-reactive skin tests, and undetectable specific IgE for *C. albicans*.

2. MATERIALS AND METHODS

2.1 Subjects

After receiving Institutional Review Board approval from the Instituto Alergoimuno de Americana (Brazil; 07/2023), we proceeded with the electronic chart review of 7,800 allergic patients who attended our outpatient facility from January 2018 to October 2023. A cohort of 100 patients had been submitted to an *ex vivo* allergen challenge test with *C. albicans* extract monitored with LAIT. The cohort counted 41 males; mean age 49,9 years; SD 18,8 years; range 10 to 88 years; modes = 25, 37, 42, 58, 60 (each appeared four times); geometric mean = 45.7 years. We offer this procedure to patients with AD with an inconclusive investigation performed with allergic skin tests and undetectable specific IgE against *C. albicans* performed with ImmunoCAP® [45].

2.2 Antigen Preparation

The strains of *C. albicans* were cultivated in Czapek medium during three weeks of incubation at 28ºC. The fungal culture was

filtered through a 0.45µm filter to obtain the fungal mass from which the micellar molecules were extracted. Extraction was performed at 4ºC for 24 hours, using a 0.125M ammonium bicarbonate extraction buffer, pH 7.5, with a high-speed stirrer. After 24 hours of extraction, the content was filtered through a coarse and 0.45 µm filter. The protein concentration was estimated spectrophotometrically and diluted to 500 µg/mL in antigen dilution 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) to perform the LAIT and allergic skin tests [45].

2.3 *Ex vivo* Investigation: Leukocyte Adherence Inhibition Test

We performed the LAIT as previously described [41,42,46-54]. Shortly, each donor's fresh plasma was divided into two parts and used in paralleled *ex vivo* challenging tests with *C. albicans* extract 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 (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, 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. 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), we subtracted the LAR from 100 (%). We employed the LAI results for the statistics calculations and the cascade distribution chart.

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 LAI mean was 41.5%; SD 29.7%, ranging from 0% to 100%; mode = 0% (appeared 17 times).

There was a wide range of distribution of LAI results, as outlined by the cascade distribution chart in Fig. 1. Seventeen patients ignored the presence of the allergen on the plasma and presented no inhibition of leukocyte adherence (LAI = 0%) after contact with the *C. albicans* extract (17 % of the tests). Some patients showed low or moderate immunoreactivity during the *ex vivo* challenge test against the *C. albicans* extract. In contrast, others displayed strong immunoreactivity that possibly would reflect the *Candida albicans* allergens' participation in the dermal inflammatory condition.

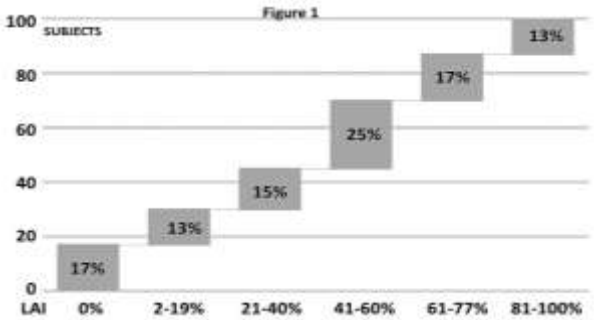

Fig. 1. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (LAI) results (x-axis %) of *ex vivo C. albicans* extract challenges monitored by the Leukocyte Adherence Inhibition Test (LAIT), according to the respective percentage of outcomes over 100 tests (y-axis)

4. DISCUSSION

AD is a multifaceted hypersensitivity condition characterized by a dysbiosis of skin mycobiome with a complex interplay between specific species of *Malassezia spp*. and *Candida spp.* with pathogenic bacteria [55]. *C. albicans* (and co-infectious microorganisms) secretes a diversity of antigens and allergens that overstimulate the immune system, increasing dermal inflammation and exacerbating AD [56]. That is why assessing *C. albicans* skin colonization concerns physicians who treat AD [57]. However, the mucosal or skin colonization *per se* does not justify the disease development since the real culprits are the individual immune responses associated with the fungal antigens [58].

Allergologists usually proceed with the propaedeutic investigation of hypersensitivity against *C. albicans* antigens in allergic patients through allergic skin tests, provocation tests, and dosage of specific IgE [59]. However, these propaedeutic resources only reflect the activity of the Adaptive arm of the Immune System. In contrast, the most significant activity against fungal antigens comes from the Immune System's Innate arm, mobilized by PAMPs and PRRs, which routine clinical laboratories hardly cover [60]. Even though the Non–IgE-mediated clinical hypersensitivities characterization is technically challenging, the novel data on basic immunology have been conceptually changing the clinical approach, liberating physicians from the IgE-limiting thought. The discovery that cytokine interactions mobilize Th2 responses independently of specific IgE-mediated reactions has provided a better understanding of Th2-associated pathologies [61]. Therefore, the finding that expression of IL-4 and IL-13 from Innate Immune System also controls type 2 immunity through Innate Lymphoid Cells was also a breakthrough in paradigmatic IgE-mediated clinical reasoning [62].

The *ex vivo* challenge test performed with the leukocyte buffy coat (as performed in LAIT) exploits various immune possibilities. This technique allows the interaction with the allergens of all blood-circulating participants, such as the innate and adaptive immune cells, cytokines, alarmins, and antibodies, covering, at least theoretically, all types of Gell & Coombs hypersensitivity reactions [63, 64]. Since the LAIT observes only the final resultant phenomenon, the leukocytes' glass-adherence inhibition after contact with tested antigens, it is not specific for any particular pathway [65-68].

This retrospective preliminary survey demonstrated, in a group of patients with AD, an extensive range of results from the *ex vivo* challenge test monitored by LAIT against *C. albicans* extract. The results suggest that most patients present some kind of immunoreactivity against their antigens, while others ignore or tolerate them. We employed LAIT as a complementary triage test to select worthwhile antigens to proceed with more laborious *in vivo* provocations when the specific IgE is undetectable. More studies with prospective larger double-blind cohorts need to evaluate the potential contribution of LAIT in diagnosing patients with *C. albicans* Non–IgE-mediated innate hypersensitivity.

5. CONCLUSION

Our preliminary results support that the LAIT may differentiate diverse degrees of *ex vivo* non–IgE-mediated innate immunoreactivity against the *C. albicans* antigens, indicating a previous immune experience with this agent. The LAIT positivity does not necessarily prove that the complaints presented by the patient while seeking medical help happened due to this specific tested antigen. The clinical diagnosis, instead, is better accomplished by the responses to the *in vivo* challenges, the degree of colonization of the patient, the benefit of an occasional pharmacological treatment, and the recrudescence of the symptoms after its interruption. More studies with prospective larger double-blind cohorts need to evaluate the potential contribution of LAIT in identifying patients with *C. albicans* Non–IgE-mediated innate hypersensitivity.

CONSENT

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

ETHICAL APPROVAL

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

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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