Original Research Article

Contribution of the Leukocyte Adherence Inhibition Test to the Diagnosis of Non–IgE-mediated Immunoreactivityagainst *Aspergillus fumigatus* in patients with Allergic Rhinitis and Asthma.

ABSTRACT

Aims: To evaluate the potential of the Leukocyte Adherence Inhibition Test (LAIT) to discriminate non–IgE-mediated immunoreactivity against *Aspergillus fumigatus* in patients with non–IgE-mediated Allergic Rhinitis and/or Asthma.
Study Design: We retrospectively examined the medical charts of 100 patients diagnosed with respiratory allergy with clinical suspicion of non–IgE-mediatedfungal hypersensitivity who were investigated with an *ex vivo* challenge monitored by LAIT against an extract of *A. fumigatus.*
Place and Duration of Study: Instituto Alergoimuno de Americana – São Paulo – Brazil – between January 2018 and January 2024.
Methodology The percentage of Leukocyte Adherence Inhibition (LAI) promoted by the *ex vivo* challenges with *A. fumigatus* extract was distributed in ranges through a cascade distribution chart to outline the variability of the results.
Results: The LAI ranged from 0% to 90%; the Mean was 52%; the Median was 55.5%; the Standard Deviation was 21%;the Mode was 61% (appeared six times), showing a Gaussian Distribution. Thisextensive distribution of LAI results suggests that some patients had non–IgE-mediatedimmunoreactivity against *A. fumigatus* allergens while others did not.
Conclusion: Our preliminary results support that the LAIT performed with *A. fumigatus* may discriminate diverse degrees of *ex vivo* immunoreactivity against this airborne allergen in patients suffering from respiratory allergies.

Keywords: Allergy; Aspergillus fumigatus; Aspergillosis; Asthma; Bronchitis; Diagnosis; Hypersensitivity; Leukocyte Adherence Inhibition Test; Non–IgE-mediated Immunoreactivity; Rhinitis.

1. INTRODUCTION

Anemophilous fungiare airborne inhabitants of thewild, rural, and urban environments able to produce allergic respiratory diseases through IgE-mediated and non–IgE-mediated hypersensitivity mechanisms[1]. Fungi are recognized as causes of reactive airway diseases such as allergic bronchitis,allergic rhinitis, allergic sinusitis, and hypersensitivity pneumonitis [2].Environmental occurrence of fungi species may produce contamination of stored food (peanuts, maize, mushrooms, rice, spices)with toxins, such as aflatoxins (named after*Aspergillus flavus*),and be highly hazardous for humans and animals [3].*Aspergillus spp*are soil saprophytic filamentous microfungi that can also be found indoors with the appearance of black molds[4].*Aspergillus fumigatus*is an airborne speciesthat can become allergenic and pathogenic upon inhalation from sensitive and immunocompromised individuals [5]. The *A. fumigatus* species comprises several strains classified in the subgenus *Fumigati*, from the genus *Aspergillus*, belonging to the family *Aspergillaceae*, from the order *Eurotiales*, from the class *Eurotiomycetes*, from the subphylum *Pezizomycotina*, from the clade *Sacharomyceta*, from the phylum *Ascomycota*, from the subkingdom*Dikarya*of the Fungi's kingdom [6, 7].*A. fumigatus* spreads through air by conidia (asexual spores)that usually does not cause damage to immunocompetent or non-sensitive subjects. However, in immunocompromised hosts, the conidia may develop into a living, invasive filamentous form,producing a respiratory disease called Aspergillosis [8].Allergic Bronchopulmonary Aspergillosis (ABPA) is an invasive clinical syndrome characterized by a peculiar combination of innate immune incompetence and IgE-Mediated

hypersensitivity that complicates previous respiratory conditions such as Tuberculosis, Bronchiectasis, Lung Abscess, and Cystic Fibrosis, clinically manifesting as asthma and respiratory failure[9]. However, *Aspergillus*-sensitizedasthma and ABPA are very distinct conditions [10]. Rarely does a patient sensitized to A. fumigatus develop ABPA. The combination of previous pulmonary damage associated with laboratory parameters such as the total IgE, the specificIgE,the *A. fumigatus*precipitins, the *A. fumigatus* skin test, the pulmonary imaging, the fractional exhaled nitric oxide, and the eosinophil count (on blood and sputum)are necessary elements to define a differential diagnosis for these conditions[11, 12].

TheAllergen Nomenclature Sub-Committee from the World Health Organization and the International Union of ImmunologicalSocieties (WHO/IUIS) had already classified dozens of allergens belonging to *A. fumigatus*[13, 14]. Several allergens and antigens of *A. fumigatus* are present in the cell wall and bind to IgE and IgG of allergic patients, producing Gell & Coombs type I and type III reactions [15]. The secretome analyses of *A. fumigatus* revealed that the Asp-hemolysin is a major secreted toxin among 63 other proteins [16]. For some time, Asp-hemolysin, a 30 KDa-glycoprotein, was thought to be the major virulent agent from *A. fumigatus*[17]. However, the main allergen (Asp f1) is a highly cytotoxic toxin with a ribonuclease activity that inhibits protein synthesis, resulting in cellular death [18]. The immunological characterization of the (yet unnominated) second major *A. fumigatus* allergen (Asp f2) suggests its role in fungal adherence and morphogenesis[19]. *A. fumigatus* also produces two forms of melanin (a complex polyketide involved in virulence and resistance mechanisms to stress): dihydroxy-naphthalene melanin (DHN-melanin) and pyomelanin [20]. The research of specific antigens to diagnose IgE-mediated hypersensitivity or IgG-mediated immunoreactivity to *A. fumigatus* is hampered by the fact that not all strains produce every specific antigen, which must be first characterized by recombinant technology before being converted into a standard diagnostic lab kit [21].

Besides their specific antigens, as any fungi, *A. fumigatus*strains also express at their cell membranes Pathogen-Associated Molecular Patterns (PAMPs), which are recognized by host innate Pattern Recognition Receptors (PRRs)[22]. The phagocytosis of *A. fumigatus* conidia by macrophages involves recognition by the Dectin-1 (beta-glucan receptor) and Toll-like receptor 2[23].Neutrophils have a critical role in host defenses against invasive Aspergillosis; however, resting conidia of *A. fumigatus* are relatively resistant to cell-free killing by oxidants generated by neutrophils [24].

The innate immune responses can develop hypersensitivity reactions by cellular activity or antigen presentation to the adaptive immune arm, which produces specific non-IgE antibodies, resulting in symptomatic clinical allergies [25]. Beyond FcεRI receptors, Mast Cells also express Fc receptors for IgG and receptors for pathogen-associated molecular patterns (PAMPs), also involved in mast cell activation and immune responses to fungi [26]. The innate hypersensitivity hypothesis was first proposed by Rajan in 2003 [27]. Fungal components can promote type IVc hypersensitivity reactions through Th17 cell cytokines and Group 3 Innate Lymphoid Cells, leading to the liberation of Neutrophil Extracellular Traps (NETs), which trap extracellular pathogens stimulating immune response and inflammation [28, 29].Nowadays, it is recognized that microbial Damage-Associated Molecular Patterns (DAMPs) are triggers of the type VII Hypersensitivity reaction through Pattern Recognition Receptors (PRRs)[30].

To evaluatenon–IgE-mediated immunoreactivity against suspected allergens, weroutinely employ the Leukocyte Adherence Inhibition Test (LAIT), an *ex vivo* immunoassay made with viable leukocytes, able to demonstrate immunoreactivity against diverse kinds of allergens, including fungi[31-36].To evaluate the potential of the LAIT to discriminate non–IgE-mediated immunoreactivity against *A. fumigatus,*we retrospectively compiled the electronic medical charts of patients with non–IgE-mediated allergic rhinitis, allergic sinusitis, and/orasthmawho were investigated with this procedure. These patients were selected after demonstrating non-reactive or inconclusive skin tests against *A. fumigatus* extract, a normal range total IgE, and undetectable specific IgE for *A. fumigatus*.

2. MATERIALS AND METHODS

2.1 Subjects

After receiving Institutional Review Board approval from the Instituto Alergoimuno de Americana (Brazil; 01/2024), we proceeded with the electronic chart review of 8,200 allergic patients who attended our outpatient facility from January 2018 to January 2024. A cohort of 100 patients had

been submitted to an *ex vivo* allergen challenge test with *A. fumigatus* extract monitored with LAIT for presenting non–IgE-mediated allergic rhinitis, allergic sinusitis, and/or asthma. The cohort counted 28 males; mean age 40.8 years; SD 21.7 years; range 2 to 81 years; modes = 51 and 58 years (each appeared five times); geometric mean = 32 years. This procedure was offered to patients with respiratory allergy with a normal range total IgE, undetectable specific IgE against *A. fumigatus* investigated through ImmunoCAP®, and a non-reagent or inconclusive investigation performed with allergic skin tests done with an *A. fumigatus* extract [37].

2.2 Antigen preparation

The strains of *A. fumigatus* 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 skin allergic tests.

2.3 *Ex vivo* Investigation: Leukocyte Adherence Inhibition Test

We performed the LAIT as previously described [32-46]. Shortly, each donor's fresh plasma was divided into two parts and used in paralleled *ex vivo* challenging tests with *A. fumigatus* 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 antigen extract (10µL of a solution with 1mg/mL and pH 7.5) or with the antigen dilution solution (when used as control). 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 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 calculate the Leukocyte Adherence Inhibition (LAI) further, 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.

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 ranged from 0% to 90%; the Mean was 52%; the Median was 55.5%; the Standard Deviation was 21%;the Mode was 61% (appeared six times), showing a Gaussian Distribution.

The cascade distribution demonstrates a wide range of distribution of LAI results (Fig.1). Two patients ignored the presence of the allergen on the plasma and presented no inhibition of leukocyte adherence (LAI = 0%) after contact with the *A. fumigatus* extract (2% of the tests). Some patients showed low or moderate immunoreactivity during the *ex vivo* challenge test, while others displayed strong immunoreactivity, which could possibly reflect the participation of *A. fumigatus* allergens in a theoreticalnon–IgE-mediated hypersensitivity condition.

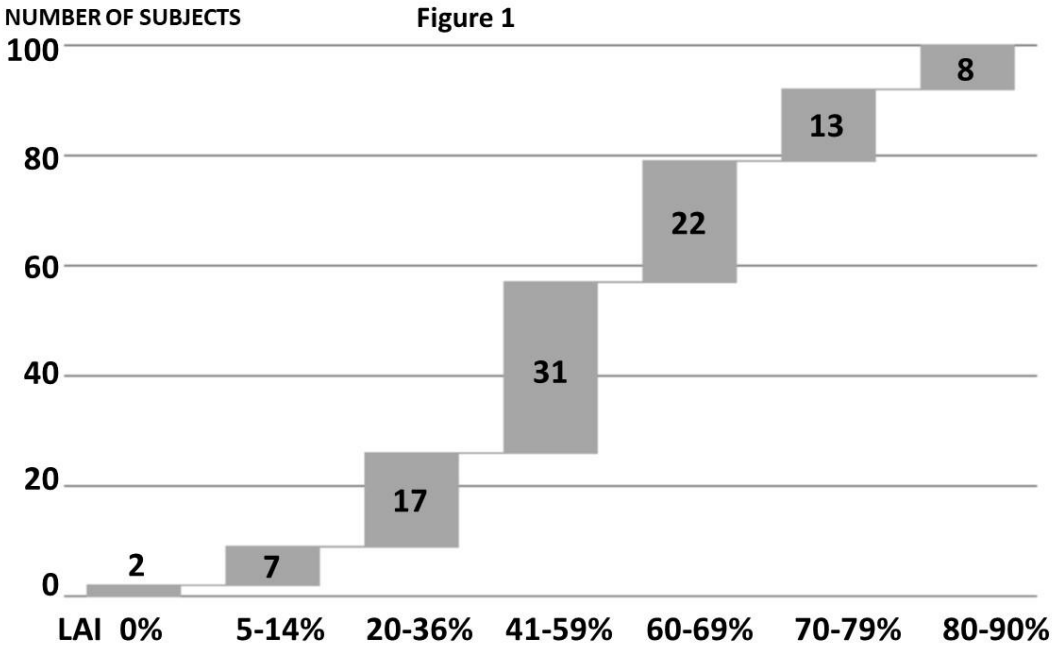

Fig. 1. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (LAI) results (x-axis %) of *ex vivoA. fumigatus* extract challenges monitored by the Leukocyte Adherence Inhibition Test (LAIT), according to the respective number of outcomes over 100 tests/subjects(y-axis).

4. DISCUSSION

Airborne allergenic fungi have been recognized as allergens by allergists and allergic patients (colloquially named "mold allergy") for a long time [47]. Respiratory fungal allergy, including allergic rhinitis, allergic bronchitis, allergic conjunctivitis, and allergic sinusitis, usually results from exposure and hypersensitivity to airborne spores with the involvement of both arms of immunity[48].

The non-IgE-mediated clinical hypersensitivities are a heterogeneous group of diseases that mainly present in common,just a technically challenging diagnosis due to the lack of an easy-to-execute, automatable,pathognomonic laboratory marker [49]. Long before the discovery of the reaginic activity of IgE, patients with asthma were classified into two overlapping categories: A) "extrinsic" when there was substantialevidence of hypersensitivity against at least one allergen, and B) "intrinsic" when there was no clear association with any suspected allergen [50]. The diagnosis of sensitization before the "IgE epoch" was performed by allergic skin tests, Complement fixation assays, and the research of precipitins [51]. Further, this classification was oversimplified by the research of specific IgE. Following this trend, the immunoassay investigation of immunoreactivity against *A. fumigatus* is mainly limited to the research of specific IgE and, sometimes, scarcely supplemented by the research of precipitins since this procedure is well-documented by the literature in patients with ABPA[52].However, the most significant activity against fungal antigens comes from the Immune System's Innate arm, represented by PAMPs and PRRs, which routine medical laboratories do not assay[53].An *ex vivo* challenge test with a viable leukocyte buffy coat can theoretically explore every immune pathway. The LAIT allows the living interaction of all immune-circulating participants with the tested allergen, such as the innate and adaptive immune cells, cytokines, alarmins, and antibodies [54].However, as an observant of the final phenomenon, the LAITdoes not indicate which pathways were involved[55-58].

This preliminaryretrospective survey demonstrated an extensive range of results from the *ex vivo* challenge test monitored by LAIT against *A. fumigatus* extract in a group of patients with AR

and/or Asthma. The condition presented by our patients is just the opposite of the conditions presented by patients with ABPA. While ABPA patients present innate immune incompetence and adaptive hypersensitivity, our patients appear to present innate immune hypersensitivity and adaptive immune incompetence to produce an active state of immunotolerance without medical help [59].

We routinely employ the LAIT as a complementary triage test to select worthwhile antigens to proceed with more laborious *in vivo* provocation tests when the specific IgE is undetectable. OurLAIT results suggest that most allergic patients present some immunoreactivity against *A. fumigatus* antigens, while some donot. However, the complete significance of these resultsis not yet fully established. As an isolated piece of information,the LAIT immunoreactivity does not prove that the complaints presented by the patient are due to the tested antigen. Indeed, the clinical diagnosis is performed by the responses to the *in vivo* challenges, the degree of colonization of the patient's environment, and the benefits of a change of ambient, an occasional antifungal or a desensitization treatment.More studies with prospective larger double-blind cohorts need to evaluate the potential contribution of LAIT in diagnosing patients with *A. fumigatus* non–IgE-mediated hypersensitivity.

5. CONCLUSION

Our preliminary results show that the LAIT may differentiate diverse degrees of *ex vivo*immunoreactivity against the *A. fumigatus* extract in patients clinically diagnosed with non–IgE-mediated fungal respiratory allergies. The propaedeutic meaning of these results, however, must be established. More studies with prospective larger double-blind cohorts need to evaluate the potential contribution of LAIT for the etiologic diagnosis of patients with fungal respiratory allergy suspected of presenting *A. fumigatus* non-IgE-mediated 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[60].

ETHICAL APPROVALS

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

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