Original Research Article

Contribution of the Leukocyte Adherence Inhibition Test to the Diagnosis of Innate Non–IgE-mediatedImmunoreactivity against *Alternaria alternata*.

ABSTRACT

Aims: To evaluate the potential of the Leukocyte Adherence Inhibition Test (LAIT) to discriminate innate immunoreactivity against *Alternaria alternata* in patients with clinical suspicion of allergic reaction to fungal allergens.
Study Design: We retrospectively examined the medical charts of 100 patients diagnosed with Allergic Rhinitis and/or Asthma with clinical suspicion of fungal hypersensitivity who were investigated with an *ex vivo* challenge monitored by LAIT against the extract of *Alternaria alternata.*
Place and Duration of Study: Instituto Alergoimuno de Americana – São Paulo – Brazil – between January 2018 and August 2023.
Methodology The percentage of Leukocyte Adherence Inhibition (LAI) promoted by the *ex vivo* challenges with *A. alternata* extract was distributed in ranges through a cascade distribution chart to outline the variability of the results.
Results: The meanLAI was 50%; SD 22,2%, ranging from 0% to 94%; mode = 58% (appeared five times).There was a wide range of distribution of LAI results, suggesting that some patients had immunoreactivity against the *Alternaria alternata* allergens while others did not.
Conclusion: Our preliminary results support that the LAIT performed with *Alternaria alternata* may differentiate diverse degrees of *ex vivo* immunoreactivity against this airborne antigen in allergic patients.

Keywords: Allergy; Alternaria alternata; Asthma; Diagnosis; Hypersensitivity; Leukocyte Adherence Inhibition Test; Non–IgE-mediated Immunoreactivity; Rhinitis.

1. INTRODUCTION

Alternaria alternata(first known as *Alternaria tenuis*) is a cosmopolitan, imperfect fungus reproducing through allergenic airborne spores responsible for several human diseases [1]. *A.alternata* is ananemophilous, melanin-pigmented fungus that forms fast-growingcolonies in dark colors ranging from gray to olivebrown[2]. *A. alternata* may exist in the soil, the atmosphere, plants, and indoor environments such as carpets, tatami mats, pillows, sofas, walls, air conditioners, dishwashers, and washing machines[3-5]. Exposure to *A. alternata*can cause allergicrhinitis, asthma, and atopic dermatitis[6, 7]. Also known as "Blackmould,"*A. alternata* is also a crop pest of tomato horticulture [8]. *Alternaria* species can also contaminate silage corn and hay, turning them into believable allergens to be liable for the historical description of "hay fever"[9]. At convenient temperatures and humidity, *A. alternata* produces mycotoxins, such as alternariol, that may impregnate food [10]. Opportunistic infestations such as chronic sinusitis and cutaneous alternariosisare described in immunodeficient patients [11, 12]. *Alternaria alternata* is the most

representativefungal aeroallergen, a target of outstanding efforts to develop new and improved desensitization techniques, such as polymerized allergoids [13]. In allergic humans, exposure to allergenic molds can cause allergic rhinitis, asthma, and atopic dermatitis[14].

The role of IL-4, IL-5, IL-9, and IL-13, along with STAT6 signaling, is a well-known mechanistic pathway leading to IgE-mediated allergic diseases [15, 16]. Besides the mainstream IgE-mediated hypersensitivity, *A. alternata* antigens can interact with innate immune cells, especially with Innate Lymphoid Cells (ILCs), increasing the inflammatory response induced by other allergens[17]. ILCs do not express lineage markers(lineage-negative);however, they can secrete cytokines that respond to pathogenic stimuli, shaping subsequent innate and adaptive Immunity [18]. *A.alternata*induces the activation of the type 2 subset of Innate Lymphoid Cells (ILC2s) that have the innate ability to express high levels of Thelper type 2 (Th2) cytokines[19]. Asthmatic patients typically present enhanced innate type 2 immune response,with almost twice ILC2s circulating in the peripheral blood compared to health controls [20]. This innate response is STAT6-dependent and leads to airway eosinophilia,peribronchial fibrosis, and thickness of the airway epithelium[21]. RAG1-deficient mice do not produce mature B and T cells [22]; however, they can generate ILC2-dependent allergen-induced memory when exposed to *A. alternata* allergens [23]. The activation of ILC2s is faster than the typical allergic response mediated by T cellsand B cells, depends on the microenvironment and cell-to-cell signals, and is independent of specific antigen stimulation[24]. Respiratory *in vivo* challenges with *A. alternata* allergens elicita rapid increase and activation of IL-33–responsive ILC2s($Lin^{-}CD25^{+}CD44^{hi}$) prepped to secrete IL-5 and IL-13, driving eosinophilic inflammation withoutB or T cells [25]. The IL-33 and its receptor (i.e., ST2) are a shared gateway for this innate pathway and its adaptive counterpart driven by allergen-reactive Th2 cells ($CD4^{+}$ Th2-type T cells), which orchestrate immune responses by the production of immunoglobulins [26]. Besides amplifying innate and Th2-type responses, IL-33 also amplifies Th1-response, enhancing the liberation of IFN-γ [27]. Full-length IL-33 is an alarmin cytokine that works as an environmental sensor, detecting proteolytic activity inside a large spectrum of allergens encompassing bacteria, fungi, mites, and pollens [28, 29].Alarmins, such as IL-33, IL-25, and thymic stromal lymphopoietin (TSLP), are ILC2-activating signals driving ILC2 growthand proinflammatory cytokine production [30].

Despite the immune blockage of IL-33 becoming a realistic target for treating refractory allergic inflammation, there is still a lack of laboratory exams to evaluate this type of immune cellular circuit activation at the clinical set [31]. When investigating an allergic patient with a clinical suspicion of hypersensitivity against *A. alternata,* the primary information that a complementary lab exam can provide is about the presence or the undetectability of specific IgE[32]. The research laboratory procedures used to elucidate innate hypersensitivity are too expensive and complex to be available to the contemporary clinical set. In search of a viable alternative, we employ the Leukocyte Adherence Inhibition Test (LAIT), performed artisanally at our modest laboratory installations annexed to our outpatient clinic. The LAIT is a simple and quick *ex vivo* laboratory procedure, made with viable leukocytes, able to demonstrate immunoreactivity against fungal allergens such as *Candida albicans* and airborne fungal allergens [33-35].

To evaluate the potential of the Test (LAIT) Leukocyte Adherence Inhibition to reproduce non–IgE-mediatedinnate immunoreactivity against the *A. Alternaria*, we retrospectively examined the medical charts of patients investigated with an *ex vivo* challenge monitored by LAIT against an*A.alternata* extract. These patients, diagnosed with allergic rhinitis or asthma,had clinical suspicion of allergic reactions to fungal allergens,had non-reactive skin tests, and undetectablespecific IgE for *A.alternata*.

2. MATERIALS AND METHODS

2.1 Subjects

After receiving Institutional Review Board approval from the Instituto Alergoimuno de Americana (Brazil; 06/2023), we proceeded with the electronic chart review of 7,700 allergic patients who attended our outpatient facility from January 2018 to August 2023. A cohort of 100 patients had been submitted to an *ex vivo*allergen challenge test with *Alternaria alternata* extract monitored with LAIT. The cohort counted 37 males; mean age 37 years; SD 24.4 years; range 1 to 88 years; modes = 31 (appeared eight times); geometric mean = 25,5 years. We offer this procedure to patients with allergic rhinitis or asthma associated with the inhalation of fungal allergens who had an inconclusive investigation performed with allergic skin tests and undetectable specific IgE against *A. alternata* performed with ImmunoCAP® [36].

2.2 Antigen preparation

The strains of *A. alternata* 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 proteinconcentration was estimated spectrophotometrically (1.36mg/mL) anddiluted to 1 mg/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) and used to perform the LAIT and allergic skin tests [37].

2.3 *Ex vivo* Investigation: Leukocyte Adherence Inhibition Test

We performed the LAIT as previously described [38-46]. Shortly, each donor's fresh plasma was divided into two parts and used in paralleled *ex vivo* challenging tests with *A. alternata* 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 mean LAI was 50%; SD 22,2%, ranging from 0% to 94%; mode = 58% (appeared five times).There was a wide range of distribution of LAI results, as outlined by the cascade distribution chart in Figure 1. Three patients (3% of the tests) ignored the presence of the allergen on the plasma and presented no inhibition of leukocyte adherence after contact with the *A. alternata*extract. Some patients showed strong immunoreactivity during the *ex vivo* challenge test against the *A. alternata* extract, which possibly would reflect the allergic symptoms after exposure to the allergen. In contrast, other patients displayed low or moderate immunoreactivity.

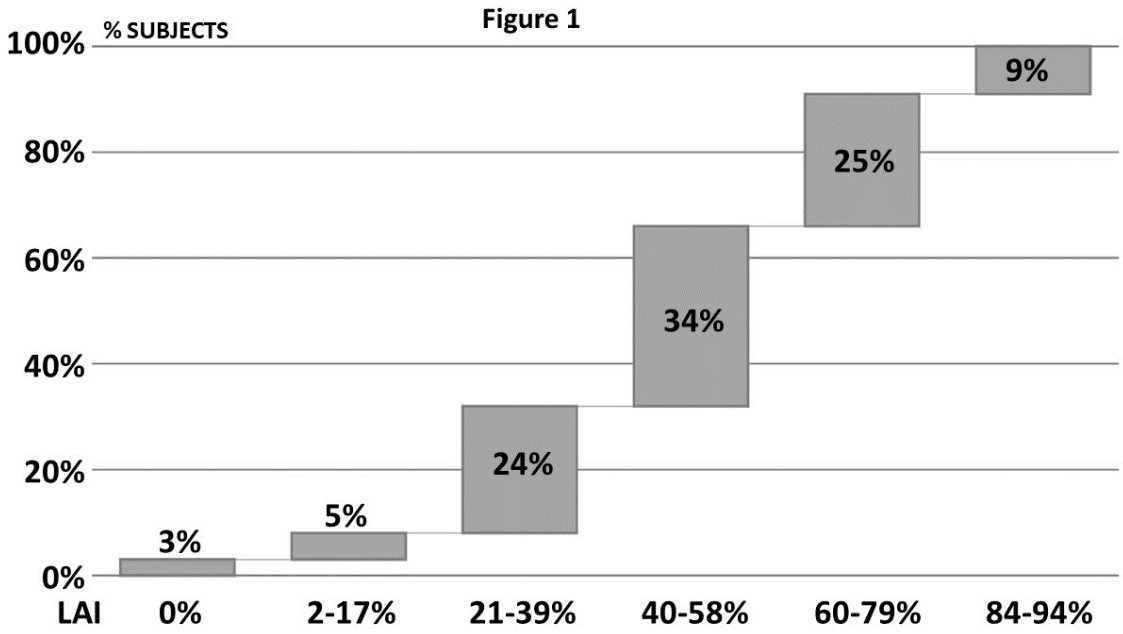

Fig. 1. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (LAI) results (x-axis %) of *ex vivoA. alternata* extract challenges monitored by the Leukocyte Adherence Inhibition Test (LAIT), according to the respective percentage of outcomes over 100 tests (y-axis).

4. DISCUSSION

The substantial improvement in knowledge about cytokine interactions from the beginning of the 21st century made us increasingly realize that IgE-mediated allergic reactions are only a tiny fraction of the complex world of hypersensitivity[47].The discovery of Innate Lymphoid Cells prepared to carry out functions formerly attributed only to the Adaptive Immune System was a paradigm shift already anticipated by several researchers [48]. The first clues about an innate cell able to generate Type 2 immune reactions derived from animal studies about immunity against helminths [49]. Tissue-resident type 2 Innate Lymphoid Cells (ILC2s) are highly responsive effectors in type 2 inflammation [50]. Soon, researchers realized the involvement of ILC2s with human allergic diseases. The ILC2s do not directly recognize specific allergens, but when stimulated by alarmins or cytokines released by damaged epithelium or neighbor immune cells, they generate Th2-type cytokines [51]. Type 2 ILCs release cytokines such as IL-4, IL-5, IL-9, and IL-13 independently of the participation of the classic Th2 cells first reported within the context of the adaptive immune system [52].As a fungus, besides the specific IgEallergens, such as the dimeric β-barrelprotein Alt a 1, *A. alternata*also possesses beta-glucans, a Pathogen-Associated Molecular Pattern (PAMP), recognized by innate receptors known as Pattern Recognition Receptors (PRRs) such as the Dectin-1, a primary β-glucan receptor,[53-56]. This innate recognition turns *A.alternata* into a perfect candidate for developing non–IgE-mediated innate Th2-like allergic reactions [57]. Besides innate cellular activation, *Alternaria*-derived serine proteasedrivesIL–33–mediated allergic inflammation [58]. The IgE-mediated hypersensitivity against *A. alternata* is easily investigated by cutaneous skin tests or by automatized ImmunoCAP® [59]. However, physicians do not readily recognize non–IgE-mediated or innate *A. alternata* hypersensitivity unless they have access to sophisticated laboratory techniques such as the Basophil Activation Test, the Lymphocyte Stimulation Test, the Leukocyte Migration Inhibition Test, or the LAIT [60-65].

The *ex vivo* challenge test performed with the leukocyte buffy coat allows the interaction of all types of peripheral blood immune cells with the allergen, theoretically exploiting an extensive range of different immune possibilities. This technique includes the participation of reactions orchestrated by alarmin cytokines, innate ILCs, and adaptive (IgE and Non-IgE) antibody-mediated reactions, classified as Types I, II, and III by Gell & Coombs, and possibly the cellular Type IV [66, 67]. The

LAIT is not specific for any pathway since it observes a final resultant phenomenon: the leukocytes' glass-adherence inhibition after contact with tested antigens[68-71].

This preliminary retrospective survey has demonstrated in a group of allergic patients a great range of results against the *ex vivo* challenge against *A. Alternaria* extract, suggesting that some patients already had a previous immunological experience with their antigens, while others did not. We employed LAIT as a complementary triage test to select worthwhile antigens to proceed with the more exhaustive *in vivo* provocations, mainly when the specific IgE is undetectable. More studies with prospective larger double-blind cohorts need to evaluate the potential contribution of LAIT in managing patients with *A. alternata*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 *A. alternata* extract, indicating a previous immune experience with this agent. The LAIT positivity does not necessarily prove that the symptoms that motivate the patient to seek 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 real-world presence of the agent in the environment inhabited by the patient, the exclusion of the allergen's source from the patient's life, and the close observance of the symptoms after its re-introduction.

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[72].

ETHICAL APPROVALS

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

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