Blocking IgE with L-glutamic acid analogs as an alternative approach to allergy treatment

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Abstract

Background: IgE–mediated allergic diseases have increased in the last decades. The most prevalent allergens from castor seeds are Ric c1 and Ric c3, isoforms of 2S albumin. These allergenic proteins cross-react with allergens from peanut, shrimp, fish, corn, gramineous, house dust, and tobacco. The usual allergy treatment employs antihistaminic and, immunotherapies. We propose an alternative immunotherapeutic approach, denoted "IgE-blockage" by L-Glutamic acid or modified-glutamic acid. Methods: Six compounds, D-glutamic acid, L-glutamic acid, N-methyl-L-glutamic acid, N-acetyl-L-glutamic acid, N-(4-nitrobenzoyl) -L-glutamic acid, and N-carbamyl-L-glutamic, were tested as a blocker. To evaluate motor coordination and the sedative/hypnotic activity of L-glutamic acid, a rota-rod test and a thiopental sodium-induced sleeping test were used. Results: the compounds, L- Glutamic acid and L-Nitrobenzoyl glutamic acid, were the most active compounds to block the interaction of castor allergens with IgE. These compounds also prevent cross-responses with allergens from food sources and inhalants that cross-react with them. In the sleeping test, the groups that received L-glutamic acid at doses of 10 and 30 mg/kg had a sleeping time similar to the vehicle control group. No changes in the animals' behavior were observed and there was no difference between the L-glutamic acid groups and the vehicle control groups in the rota-rod test. Conclusion: L-glutamic acid and L-Nitrobenzoyl glutamic acid could be used as IgE blockers to prevent allergic diseases.

Introduction

Allergy mediated by Immunoglobulin E (IgE) including, asthma and severe food allergy, has significantly increased in many countries in the last decades and has become a worldwide public health issue[1,2]. IgE-mediated hypersensitivity reactions are characterized by mast cells' activation and tissue infiltration, as well as activation of inflammatory cells [3]. Atopy results of the interplay between genetic and environmental factors, thus when atopic individuals are exposed to allergens, sensitization occurs in a T-helper- type-2(Th-2) dependent pathway, that is characterized by the production of several cytokines, mainly interleukin(IL)-4 and IL-13 causing the generation of allergen-specific IgE antibodies by plasma cells [3,4]. IgE antibodies generated, in response to a specific allergen, interact with this allergen triggering a series of intracellular reactions leading to the release of histamine and other inflammatory mediators [5,6]. The release of histamine causes smooth muscle contraction of the gastrointestinal tract and respiratory tract, nerve stimulation, and vasodilatation[5,7]. Specific-IgE generated in response to a determined allergen may cross-react with proteins sharing structural homology in the amino acid sequence with the original immunogen[8,9]. The reserve proteins, belonging to 2S albumin class are the major allergens present in the seeds[9]and can cause cross allergic reactions in previously sensitized individuals [10]. Since the identification of the critical role mediator of the IgE in allergic diseases, the control of the IgE responses has become one of the main therapeutic objectives[11]. Strategies for treatment of allergy widely studied, such as pharmacotherapy, immunotherapy, and anti-IgE therapy, have gained special attention after understanding the clinical importance of the IgE antibody's specific activity [12]. The antihistaminic drug is the most common treatment for allergy, it binds to histamine

receptors and inhibits their effects, but this drug does not address the leading cause of allergic responses, it only alleviates the symptoms [7]. In order to develop other therapeutic approaches for allergic disorders, new effective, and safe therapies for allergy treatment are needed [13,14]. Omalizumab (Xolair)-based anti-IgE therapy binds free, but not FcεRI-bound IgE, which is mostly used for the treatment of asthma and urticaria. Besides, the requirement for many doses and the medicine high cost limits the extensive use of this treatment [6]. We propose an complementary immunotherapeutic approach, denoted "IgE-blockage" [15]. This proposal is based on the interaction of free amino acid with Fab-IgE, blocking the allergen recognized by IgE. Studies developed by Deus de Oliveira *et al* ., 2011[16] identified amino acids, such as, glutamic acid and aspartic acid present in the IgE binding epitopes from major allergens from castor seeds, Ric c1 and Ric c3 [16]. Free Glutamic acid (Glu), when incubated with serum anti–castor allergens works as IgE binding blockers, it prevents mast cell degranulation and subsequent histamine release [15,16]of the activated mast cells. Here, we evaluated the use of L-Glu and analog amino acid as IgE-blocker. In addition to the possible effect on the inhibition of allergy. The effect of treatment with these compounds on neurological functions will also evaluated it through behavioral tests.

Materials and Methods

Plant material and 2S albumin purification

Castor seeds (*R. communis* L., cultivar IAC-226) were obtained from the Instituto Agronômico of Campinas, São Paulo/ Brazil. The 2S albumin fractions were isolated and characterized by SDS-PAGE and immunoblotting experiments, as described previously by Deus-de-Oliveira et al., 2011[16].

Drugs and chemicals

The drugs and chemicals used for the experiments were diazepam 5 mg/Kg (Hipolabor Pharmaceutical, Brazil), thiopental sodium 40 mg/Kg (Cristália, SP, Brazil). The amino acids D-glutamic acid, L-glutamic acid, N-methyl-L-glutamic acid, N-acetyl-L-glutamic acid, N- (4-nitrobenzoyl) -L-glutamic acid, and N-carbamyl-L-glutamic acid were from Sigma.

Experimental animals

Eight-week-old female Balb/c mice were purchased from the animal facility of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF). All experimental procedures complied with the Ethical Commission in Animal Experimentation of the UENF (Proc. CEUA-UENF/297), which fulfills the principles of ethics for animal research adopted by the National Council for Control of Animal Experimentation (CONCEA). Mice were housed in appropriate conventional animal care facilities and handled according to international guidelines for animal experiments. The animals were divided into control and test groups containing six mice each.

Mice sensitization

Briefly, BALB/c mice were sensitized three times (at days 0, 7, 14, 21, and 28) intraperitoneally (i.p.) with 2S albumin from castor seeds. Two concentrations of allergenic protein were used, 1 and 10 µg in 200 µL per injection, at intervals of one week. The allergen was emulsified with 5 mg aluminum hydroxide gel (4 mg/mL) (Sigma, São Paulo, SP). After the third immunization (day 21), the animals received a booster with of the antigen in the same concentration in the presence of 2,5 mg aluminum hydroxide gel (4 mg/mL), and this procedure was repeated at day 28 without adjuvant only using 2S albumin at concentration 50 µg in 200 µL per injection. The mice were anesthetized using Anestalcon® 5,0 mg/mL (0.5% Proximethyl chloridrate) and bled. The bleeding technique was by retro-orbital plexus.

Serum immunoglobulins detection

Polystyrene 96-well plates (Nunc-Immuno Plate I F) were coated with purified 2S albumin 60 µL/well (1 µg/µL in sodium carbonate-bicarbonate buffer 50 mM pH 9.6) at 4 °C overnight. After washing with PBS containing 0.05% Tween 20 (PBS-T), the plates were blocked with PBS-T containing 1% gelatin for one hour at room temperature, washed with PBS-T. Washed with PBS-T and incubated with serum samples

diluted in 0.1% PBS-T gelatin at 37 °C for one hour. After, the wells were washed with PBS-T and treated with peroxidase-conjugated goat anti-mouse (Southern Biotech) IgE or IgG or IgG1 antibodies (diluted according to manufacturer's instructions) in PBS-T 0.1% gelatin for one hour at 37°C and were washed. An o-Phenylenediamine (OPD) peroxidase substrate (Sigma) solution was added, and the color development by 15 min. The reaction was stopped using sulfuric acid 3M, and colorimetric intensity was measured by absorbance 495 nm using a Microplate Reader (Thermo Plate).

Cross-reactivity

To investigate cross-reactivity between 2S albumin from castor seeds and food or aeroallergens used for allergy diagnosis, the plates were previously sensitized with 60 µg of airborne allergens (FDA Allergenic) or food allergens (FDA-FOOD KIT), both solutions containing solution (10 µg/mL). Cross-reactivity was determined by ELISA, as previously described.

Effect of glutamic acid and analogs as an IgE blocker

The ability of L-glutamic acid and analogs as D-glutamic acid, N-methyl-L-glutamic acid, N-acetyl-L-glutamic acid, N- (4-nitrobenzoyl) -L-glutamic acid, and N-carbamyl-L-glutamic acid to inhibit the binding of specific IgE to allergens was assessed by ELISA inhibition. These amino acids were prepared in saline solution at concentrations of 0.5 µM. Serum pool from animals sensitized against 2S albumin from *R. communis* (1:5 diluted) was pre-incubated for 20 minutes with glutamic acid, and their analogs at end concentration 0.05 µM (10.µL amino acid solution + 90 µL of diluted serum). The ELISA assays were performed as previously described. The percentage of IgE binding inhibition achieved by the pre-incubation treatment was calculated as follows: percentage of IgE binding =100–(ODI/ODT X 100); ODI represents the absorbance after incubation of glutamic acid-treated human serum, and ODT represents the absorbance of untreated animal serum. Also, the serum pool of specific IgE 2S anti-albumins was pre-incubated with increasing concentration at end concentration 0,05 to 0,01 µM of each amino acid.

Isolated aspartic acid was also incubated with the serum pool of patients allergic to *R. communis* . Additional controls were employed. As a negative control, an extract from egg allergens, which did not cross-respond with allergens from *R.comunis* , was included. To evaluate the specificity of glutamic acid or aspartic acid, a pool of protein amino acids, excluding glutamic acid and aspartic acid, was also tested as a blocker.

Thiopental sodium-induced sleeping time test

The method employed in this study was described by Sharmen *et al.* 2014[17]. The animals were randomly divided into five groups consisting of six mice each. The test groups received L-glutamic acid at the doses of 10, 30, and 50 mg/kg (i.p.), while the positive control group was treated with diazepam (5 mg/kg; i.p.) and negative control group with vehicle (0.9% physiological saline; i.p.). Thirty minutes later, thiopental sodium (40 mg/kg; i.p.) was administered to each mouse to induce sleep. Sleeping time was calculated as the interval between the loss and the recovery of the righting reflex.

Motor test

The motor coordination effect was assessed using a Rotarod apparatus. Experimental animals were subjected a pretest in which the animals were trained on the apparatus during two days before experiment. Only those animals, which demonstrated their ability to remain on the revolving rod, were used. On the day of the experiment, the test groups received L-glutamic acid at the doses of 10, 30, and 50 mg/kg (i.p.), whereas the negative control group received vehicle (0.9% physiological saline; i.p.). Animals in the positive control group received diazepam (5 mg/ kg; i.p.). Thirty minutes after administering drugs, each mouse was placed on the rotating rod (rotational speed of 20 RPM) for five minutes (300s). Time spent in the apparatus was observed for 5 min duration (300 s).

Statistical analysis

Experimental values were expressed as means +-SD. Data were analyzed using one-way analysis of variance, or where applicable ANOVA followed by post hoc analysis with Duncan comparison test. The P values were

considered significant if p< 0.05 or p >0.05. The analysis was performed using GraphPad 5 software.

Results:

Evaluation of immunoglobulin profile (IgE, IgG, and IgG1)

The immunoglobulins profile obtained after 2S albumin (Ric c1 + Ric c3) immunization is presented in figure 1. The presence of IgG1 and IgE characterize the allergic response.

The levels of each immunoglobulin were slightly higher when immunization was performed with 10 μg protein than immunizations with 1 μg.

Glutamic acid protects against cross-reactivity

The pre-incubation of serum with glutamic acid promoted inhibition of IgE reactivity to both airborne and food allergens, indicating that the carboxyl group of these amino acids could be significant in IgE-epitope interactions. Lower blockages were observed for gramineous and peanut (Figure 2).

IgE blocking assays using modified glutamic acids.

The Figure 3 showed that, among the amino acids evaluated as a possible blocker, L-glutamic acids (60% of blockage) and N- (4-nitrobenzoyl) -L-glutamic acid (93% of blockage) are the best compounds for protection of the interaction between IgE and allergenic proteins (Ric c1 + Ric c3). N-carbamyl-L-glutamic acid (42%) and N-acetyl-L-glutamic acid (36%) could be used as IgE blockage, however N-methyl-L-glutamic acid blocker (E2) (25%) and D-glutamic (14%) presented a low percentage of IgE blockade. No IgE block was observed when a pool of amino acid, excluding dicarboxylic amino acids, was employed.

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The dose-response curve of blocking agents.

Figure 4 shows the influence of modified glutamic amino acid concentration to block IgE-2S albumin interaction, investigated by ELISA. Best results were observed when L-Glutamic acid and N- (4-nitrobenzoyl) -L-glutamic acid (E4) were used. These amino acids promoted ˜100% IgE blockade in the more significant tested concentration. D-Glutamic acid (E1) and N-carbamyl-L-glutamic acid (E5) blocked ˜40%. However, when N-acetyl-L-glutamic acid (E3), N-methyl-L-glutamic acid (E2), were used, lower levels of blockage (˜20%) were observed.

Behavioral evaluation of animals after treatment

Motor test

The residence time in the Rota-Rod apparatus after treatment with glutamic acid, at the tested concentration (10, 30, or 50 mg/kg) presented a higher time of permanence in the apparatus than the group of Diazepam (Figure 5), similar to the control (animal treated with vehicle).

Sleeping Time Test

The effect of the glutamic acid (10 mg/kg or 30 m/kg) on the onset of sleep were comparable to that of standard (vehicle). A significant reduction in the time of onset of sleep in a dose-dependent manner was observed with glutamic acid at 50 mg/kg (Figure 6).

Discussion

Understanding the clinical importance of the IgE antibody's specific activity has a crucial role in developing new therapeutic strategies for the treatment of allergy. This immunoglobulin has a crucial role in immune response as allergic diseases. Moreover, the frequency of IgE–mediated allergic diseases has significantly increased in the last decades, amplifying the concern on developing preventive and alternative approaches for implementing novel treatment strategies to control allergic disorders [18.]

The importance of glutamic acids in the formation of IgE-binding epitopes was demonstrated for two allergenic isoforms, Ric c 1 and Ric c 3, both from castor 2S albumin [15,16]. Castor 2S albumin can cross-react with allergens from shrimp, fish, corn, wheat, soybean, peanut, house dust, tobacco, and airborne fungi. Thus, the exposure to the allergens presents in pollen or seeds of castor can become an individual sensitized and can trigger an allergic response when exposed to other allergenic sources. It was found that the L-Glu could be blocking the cross-reaction between castor 2S albumin and peanut, shrimp, fish, corn, gramineous, house dust, and tobacco (Figure 2).

As proposed by Aswar *et al.* 2015 [18], we investigated if the possible therapeutic doses could induce behavioral alterations in motor test and sleeping time. A decrease in locomotor activity indicates a sedative effect [19] as observed for diazepam use [24]. In all doses tested, 10, 30, and 50 mg/kg of body weight, L-Glu showed performance similar to a positive control (vehicle) in a locomotor score (figure 5). Doses of 10 mg/kg or 30 mg/kg of this amino acid do not cause sedative activity; however, a decrease in the potentiation of hypnotic effect induced by the thiopental sodium was observed in doses of 50 mg/kg of L-Glu.[20] Alternative compounds, derived from glutamic acid, were also capable of preventing allergen's binding, castor albumin 2S, as N-(4-nitrobenzoyl) L-glutamic acid that blocked 100% the binding of allergen to IgE (Figure 3). The binding between IgE and L-Glu or derivatives, occurred at pH 7.0, a physiological pH, probably due to electrostatic interaction. In this pH, the amino acid Glutamic carries negative charges from the carboxyl groups thus can form ion-pairs with the positively charged amino acid residues on the IgE molecules. Hubbard et al. 2013[21] demonstrated that Molecular recognition between antibody and antigen also involves interactions between surfaces that are continuously in movement, and binding reactions may involve some conformational changes by the antigen or antibody before the formation of the antigen-antibody complex [22.] We observed that D-glutamic does not block the interaction between allergen and IgE, suggesting a stereo-selectivity for this interaction. Other L-modified glutamic acids as N- (4-nitrobenzoyl) -L-glutamic acid and N-carbamyl-L-glutamic acid could also be an IgE blocker; However, animal behavior studies are required for the indication of these compounds for begging purposes. In conclusion, the blocking IgE by glutamic acid-free may be an approach for allergy treatment, although more in vivo studies are needed.

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Disclosures

None of the authors have financial or commercial conflicts of interest.

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Figure legends

Fig.1: Detection of immunoglobulins in the serum of mice sensitized with 2S albumin, 1 and 10 µg/ 200 µL. Primary antibody: 1:5 (IgE); 1:500 (IgG and IgG1). Anti-IgE: 1:2000 (IgE); The means for six mice per group are shown.

Fig. 2: Effects of blocking modified glutamic acids on IgE binding to albumin 2S in ELISA. A serum without treatment was used as the control (100% of mast cell degranulation. L- Glutamic acid (Glu), D-Glu (E1), N-methyl-L-glutamic acid (E2), N-acetyl-L-glutamic acid (E3), N- (4-nitrobenzoyl) -L-glutamic

acid (E4), and N-carbamyl-L-glutamic acid (E5) were each mixed with a diluted pooled plasma at a ratio of 1:10 (v:v), and incubated in a micro plate coated with 2S albumin. IgE binding was detected using a Goat anti-mouse IgE HRP conjugate (1:2000) and an OPA substrate. Absorbance was read at 492 nm. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-test. * P<0,01 e * * P<0,05.

Fig. 3: Blocking profile of IgE binding to 2S albumin protein by ELISA. Total binding was determined by binding of immune serum anti-2S albumin incubated with: Glu, L-Glutamic acid; E1, D-glutamic acid; E2: acid-N-methyl-L-glutamic acid; E3 acid - N-acetyl-L-glutamic acid; E4, acid - N- (4-nitrobenzoyl) - L-glutamic acid; E5, N-carbamyl-L-glutamic acid. All amino acid derivatives at concentration of 0.5 µM diluted 1:5 in immune serum. Microplates were coated with albumin 2S protein (20 µg). The IgE binding was detected, using a Goat anti-mouse IgE HRP conjugate (1: 2000) and a colored substrate. Absorbance was read at 492 nm. The Mean values from 3 assays?$_{??}$SD are presented

Fig. 4: Blocking profile of IgE binding to castor-2S albumin and allergens from house dust, gramineous, tobacco, peanut, corn, and fish quantified by ELISA. Immune 2S anti-albumin serum diluted (1:5) was treated with L-glutamic acid before immune assay. Microtitre plates were coated with 10 µL of allergens solutions (0.5 µM). The IgE binding was detected using a Goat anti-mouse IgE HRP conjugate (1: 2000) and a colored substrate. The Mean values from 3 assays?$_{??}$SD are presented

Fig. 5: Evaluation of the permanence time of mice treated with Glutamic Acid in the Rota-Rod apparatus after treatment with glutamic acid 1. Vehicle (saline solution); 2. Diazepam, 3. Glutamic acid (10 mg / kg); 4. glutamic acid (30 mg / kg); 5. glutamic acid (50 mg / kg). The means for six mice per group are shown. In comparison with DIAZEPAM, by one way ANOVA * P <0.01 and * * P <0,05.

Fig. 6: Evaluation of the potentiation effect of sleep time by sodium thiopental (TPT) on the induction of sleep time in mice after treatment with glutamic acid. 1. Vehicle (saline solution); 2. Diazepam, 3. Glutamic acid (10 mg / kg); 4. glutamic acid (30 mg / kg); 5. glutamic acid (50 mg / kg). Statistical analysis was performed using one-way ANOVA followed by Duncan post-test. * P<0,01 e * * P<0,05