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RESEARCH ARTICLE

Effectiveness of Shrimp Allergenic Extract as an Immunotherapy Agent in Mice Model of Gastrointestinal Allergy

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ABSTRACT:

Allergen extract as allergen-specific immunotherapy (AIT) is the only causative therapy and provides protection or tolerance to an allergen in the long term. However, allergen extracts from different countries may have different effectiveness. This study aimed to evaluate the effectiveness of Indonesian shrimp allergen extract (SAE) as an immunotherapy agent with a mouse model of allergies in the gastrointestinal tract. Mice were divided into five groups consisting of the naïve group, allergic group, and the allergic group received SAE immunotherapy at high dose (100μg/week), moderate dose (50μg/week), and low dose (10μg/week). Each group received treatment in the sensitization and desensitization phases, which was then followed by an oral challenge of SAE 100μg. The effectiveness of SAE immunotherapy was assessed based on the parameters of systemic allergic symptoms, IL-10 mRNA expression in ileum tissue, and IgG2a serum concentration. We found that SAE immunotherapy decreased the systemic allergic symptoms score, regardless of dosage, and the effect persisted on the third challenge. IgG2a as a parameter of humoral immunity showed a significant increase in the high-dose immunotherapy group, and IL-10mRNA expression as a parameter of cellular immunity also showed an increase in the high-dose group. Both data showed a dose-dependent manner. It can be concluded that SAE has excellent effectiveness as an immunotherapy agent and dose-dependent characteristics.

KEYWORDS: Gastrointestinal allergy, Allergenic Shrimp Extract (ASE), Allergen specific-immunotherapy (AIT), IL-10, IgG2a, Neglected disease.

INTRODUCTION:

Food allergy was one of the allergic diseases that is recognized as a public health burden[1]. In Asian countries, shellfish allergy was one of the most common food allergies[2], with a prevalence up to 5.2%[3], and its prevalence among children was estimated to increase by 1.2% each decade[4]. There were no drugs intended to prevent food allergic reactions, especially in Indonesia. The medications used were generally symptomatic. These symptomatic drugs were known to have a good effect in relieving immediate allergic symptoms, but not to prevent allergy relapse and need to be aware of the side effects if used long term[5]. For this reason, allergy therapies that were causative and/or provided long-term protective effects/tolerance to allergens were needed.

Shrimp allergen extract (SAE) as allergen-specific immunotherapy (AIT) is the only therapy to modify allergic disease. Strategies applied by gradual induction of immune tolerance (desensitization) to specific allergens[6,7]. Therefore, allergen-based immunotherapy was recommended for allergic patients as an alternative to allergy therapy for long-term treatment. Administration of allergen extract-based immunotherapy can induce immune tolerance. The treatment will

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prevent the recurrence of allergy diseases such as rhinitis, asthma, and urticaria[8,9]. However, allergen extracts from the same source but produced by different manufacturers can also have very different compositions and potencies. These facts are due to the wide range of allergen content permitted by The Monograph of Allergen Products (50-200%, tested by IgE inhibition test)[10,11].

Therefore, an evaluation of the effectiveness of the Shrimp Allergenic Extract (SAE) which is being developed in Indonesia, is evaluated on a gastrointestinal allergic mouse model. The approach used to observe the effectiveness of SAE included systemic allergic symptoms, serum IgG2a concentrations, and IL-10 mRNA expression in the gastrointestinal model of mice.

MATERIALS AND METHODS:

Materials:

Balb/c mice were acquired from Farma Veterinary Canter, Surabaya, Indonesia. The animal protocol was approved by the Animal Care and Use Committee, Faculty of Veterinary Universitas Airlangga Surabaya. Shrimp Allergenic Extracts (SAE) were obtained from Dr. Soetomo Teaching Hospital, Surabaya, Indonesia. Aluminum hydroxide (Alum) was purchased from Merck KGaA, Darmstadt, Germany. IgG2a ELISA kit from MyBioSource, San Diego, USA. Invitrogen™ PureLink™ RNA Mini Kit from Thermo Fisher Scientific, Waltham, MA, USA. QuantiFluor® RNA System, GoScript™ Reverse Transcription System, and GoTaq® qPCR Master Mix from Promega, Madison, WI, USA. IL-10 primer (5'-CAGTACAGCCGGGAAGACAATA-3') from Macrogen, Seoul, South Korea.

Methods:

Mouse Model of Gastrointestinal Allergy and SAE Immunotherapy:

Female BALB/c mice (aged 4-5 weeks) were maintained on a shrimp-free diet. The treatment groups were sensitized with 100μg SAE in 200μl Saline intraperitoneally together with 1mg of Alum as an adjuvant on days 0, 7th, and 14th (sensitization phase). For the naïve group (N), mice were given 1 mg of Alum in 200μl Saline[12,13]. On the 20th day, mice were fasted overnight and challenged with 400μg SAE orally on the 21st day. This step was repeated three times to ensure that the allergy model formed. On the day 32nd, 39th, and 46th (desensitization phase), mice in the immunotherapy groups were administered with 100μg (high dose group/HI), 50μg (moderate dose group/MI), and 10μg (low dose group/LI) of SAE in 200μl Saline intraperitoneally; and mice in the negative control (NC) and naïve (N) groups were administered with 200μl saline intraperitoneally. On the 52nd day, mice were fasted overnight and challenged with 400μg SAE orally on the 53rd day to evaluate the effectiveness of SAE immunotherapy. On the 57th day, mice were fasted overnight and challenged with 400μg SAE orally on the 58th day to assess whether the efficacy of SAE immunotherapy persisted five days after the last challenge. On the 59th day, blood serum samples were collected to analyze IgG2a serum levels, and small intestine tissues were collected for analysis of IL-10 mRNA expression (figure 1).

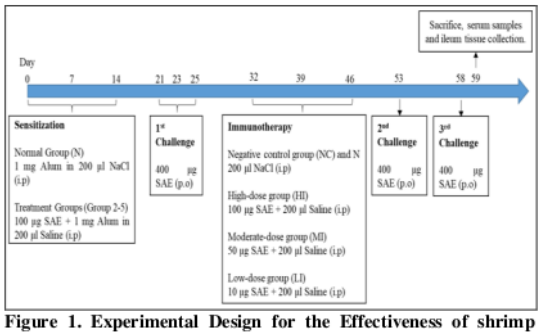

Figure 1. Experimental Design for the Effectiveness of shrimp allergenic extract as an immunotherapy agent

Assessment of Systemic Allergic Response:

Systemic allergy response assessment was carried out by placing the mice in individual cages and recording the mice's activity for 30 minutes after the challenge. Scoring response as described: 0, no symptom; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and snout, reduced activity and/or decreased activity with the increased respiratory rate; 3, wheezing, labored respiration, cyanosis around the mouth and the tail; 4, no action after prodding, or tremor and convulsion; 5, death[14].

Measurement of Serum IgG2a Levels:

Serum levels of IgG2a were measured by ELISA as described in the manual of IgG2a ELISA Kit (MyBioSource). Briefly, 100μl of each sample, standard, and blank solution, has been prepared before, were added into the defined antibody-coated well, and incubate at 37°C for one h. Then, the wells were washed and followed by the addition of biotinylated detection antibody, HRP conjugated antibody, and developed as described above. The wells were washed, followed by the addition of 90μl TMB substrate, and incubate at 37°C for 15 m. When there were color changes, the reactions were stopped by adding 50μl stop solution into the wells. The microplate was inserted into the ELISA reader to measure the absorbance at 450nm from each well.

IL-10 mRNA Expression in Ileum tissues:

Total RNA was extracted using Invitrogen™

PureLink™ RNA Mini Kit, the concentration and purity of purified total RNA were determined using QuantiFluor® RNA System (Promega). cDNA was synthesized from the RNA using GoScript™ Reverse Transcription System (Promega). PCR was performed on the MyGo Mini 16 Sample 2-Plex Realtime PCR System (IT-IS Life Sciences) under condition as described: hold, 95°C for 120s; 2-step amplification, 45 cycles of 95°C to 60°C; high resolution melting, 60°C to 97°C at 0,05°C/s. Expression quantification analysis by comparing relative to mRNA with a cycle threshold method. Endogenous gene reference using β-actin.

RESULT:

Sensitized mice exhibited systemic allergy responses to SAE:

Observation of systemic allergy symptoms is one model of behavioral observation that represents the clinical manifestations of food allergy, which is widely reported as a systemic anaphylactic response[1]. Administration of SAE 100μg in all treatment groups showed a significant increase in systemic allergy symptoms compared to the naïve group (p<0.01 and p<0.001). These three times challenges in the sensitization phase did not cause desensitization (figure 2).

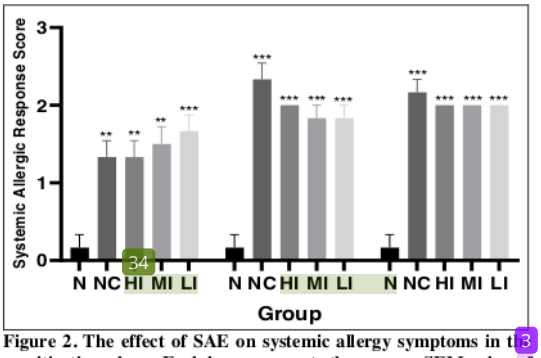

Figure 2. The effect of SAE on systemic allergy symptoms in the sensitization phase. Each bar represents the mean ± SEM value of 6 mice/group. **p<0.01 and ***p<0.001 compared to normal group. Statistical tests were performed using the Kruskal Wallis test. N = normal, NC = negative control, HI = high dose immunotherapy, MI = moderate dose, LI = low dose group.

SAE immunotherapy reduced systemic allergy responses:

Systemic allergy symptoms were also observed after mice were given immunotherapy to determine the effectiveness of SAE as an immunotherapy agent. The findings in this study showed that there was a significant difference in the negative control group and the low dose group compared to the naïve group (p<0.01 and p<0.05). However, as for the immunotherapy group, a significant decrease in systemic allergy symptoms only occurred in the high-dose immunotherapy group when compared to the negative control group (p<0.05) (figure 3).

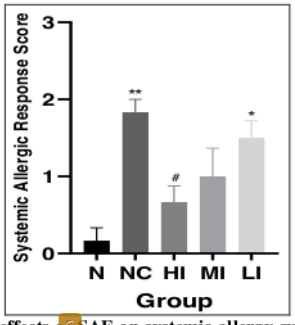

Figure 3. The effects of SAE on systemic allergy symptoms in the desensitization phase. Each bar represents the mean ± SEM value of 6 mice/group. **p<0.01 and *p<0.05 compared to the N group, #p<0.05 compared to the NC group. Statistical tests were performed using the Kruskal Wallis test. N = normal, NC = negative control, HI = high dose immunotherapy, MI = moderate dose, LI = low dose group.

SAE immunotherapy was still influential on the fifth day after the last challenge:

In addition, to evaluate whether the efficacy of SAE immunotherapy persisted for a specific duration of time, the third challenge is carried out, and systemic allergy symptoms are observed again on the fifth day after the last challenge. The results obtained showed that there was a significant difference in the negative control group (NC) compared to the naïve group (p<0.001). Furthermore, the results obtained in the high and moderate dose immunotherapy group showed a significant decrease in systemic allergy symptom scores compared to the negative control group (p< 0.01 and p< 0.05) (figure 4).

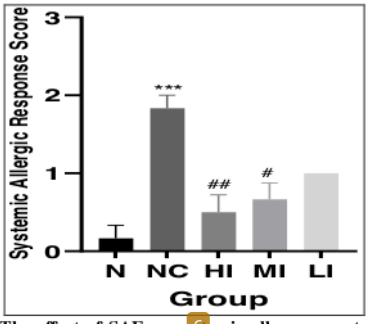

Figure 4. The effect of SAE on systemic allergy symptoms on the fifth day after the last challenge. Each bar represents the mean ± SEM value of 6 mice/group. ***p<0.001 compared to the N group, ##p<0.01 and #p<0.05 compared to the NC group. Statistical tests were performed using the Kruskal Wallis test. N = normal, NC = negative control, HI = high dose immunotherapy, MI = moderate dose, LI = low dose group.

High dose SAE Immunotherapy resulted in the increase of IgG2a serum levels

In this study, serum levels of IgG2a, as a systemic marker related to the immune tolerance system due to SAE immunotherapy in allergic diseases, were measured

using the enzyme-linked immunosorbent assay (ELISA) method. A significant increase in IgG2a serum levels only occurred in the high-dose immunotherapy group compared to the naïve group ($p<0.05$) and the negative control group ($p<0.05$) (figure 5).

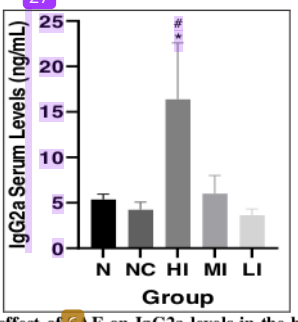

Figure 5. The effect of SAE on IgG2a levels in the blood serum of experimental animals. Each bar represents the mean ± SEM value of 3 mice/group. *$p<0.05$ for the N group, #$p<0.05$ for the NC group in the desensitization phase. Statistical tests were performed using one-way ANOVA. N = normal, NC = negative control, HI = high dose immunotherapy, MI = moderate dose, LI = low dose group.

High dose SAE Immunotherapy increase IL-10 mRNA expression in Ileum tissues:

Measurement of IL-10 mRNA expression in ileal tissue (as a local marker related to the immune tolerance system due to SAE immunotherapy in allergic diseases) was carried out using the Real-Time Polymerized Chain Reaction (RT-PCR) method. This measurement was carried out to determine the effect of SAE immunotherapy on IL-10 mRNA expression. A significant increase in IL-10 mRNA expression was found in the high-dose SAE immunotherapy group compared to the naïve group ($p<0.05$). At moderate and low doses, the SAE immunotherapy group tended to have higher IL-10 mRNA expression values than the naïve and negative control (figure 6).

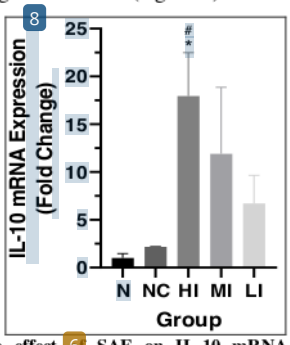

Figure 6. The effect of SAE on IL-10 mRNA expression of experimental animals. Each bar represents the mean±SEM value of 3 mice/group. *$p<0.05$ for the N group, #$p<0.05$ for the NC group in the desensitization phase. Statistical tests were performed using one-way ANOVA. N = normal, NC = negative control, HI = high dose immunotherapy, MI = moderate dose, LI = low dose group.

DISCUSSION:

Food allergy is one of the allergic diseases that is recognized as a public health burden, especially in developing countries, with a reasonably high global prevalence and increasing every decade and is referred to as the second wave of allergies, after asthma[1,17]. In food allergies, symptoms of hypersensitivity reactions would be occurred if influenced and exacerbated by several factors, including allergens in the food itself, the presence of infectious agents such as toxins[18,19]. Several studies show different clinical manifestations of food allergy in certain age groups. One of the conditions that can be found in cases of food allergies is gastrointestinal tract disorders[20]. Allergic symptoms are caused by repeated exposure to allergens that cause an immediate allergic reaction and activation of allergen-specific T cells (T helper 2/Th2) cells leading to eosinophilic associated gastrointestinal disorders/EGIDs[21,22]. To further study the mechanism of a systemic allergic response and develop effective immunotherapy regimens for shrimp allergy, valid animal models are needed. This study showed that systemic allergic response, including increased rubbing scratching frequency, changed the morphology of snout, reduced activity with and/or without increased respiratory rate, and wheezing or labored respiration, could be induced wheezing or labored intraperitoneal respiration sensitization of BALB/c mice with SAE. In this study, we examined the effectiveness and dose-response of SAE immunotherapy in the mice model of shrimp allergy. In contrast, the previous studies showed that most shrimp-allergic patients react to tropomyosin[14,23] and sublingual immunotherapy of shrimp extract[24]. Our findings showed that SAE immunotherapy reased systemic allergic response dose-dependently compared to the negative control group. The decrease in systemic allergic symptoms that occurred due to the use of SAE immunotherapy in a mouse model correlated with a reduction in systemic allergic symptoms in human[25]. It means that SAE was effective as an immunotherapy agent based on the decrease in allergy symptom response. In this study, systemic allergy symptom scores were also observed five days after the last challenge. The results showed that the administration of SAE immunotherapy reduced the score of systemic allergy symptoms. The data indicate that SAE immunotherapy still had good effectiveness during this period.

In food allergy, the balance between the production of allergen-specific Immunoglobulin E (IgE) and blockade by Immunoglobulin G (IgG) determines the occurrence of allergic symptoms. This study measured serum levels of IgG2a, as a systemic marker related to the immune tolerance system due to SAE immunotherapy in allergic diseases, using the enzyme-linked immunosorbent assay (ELISA) method. This study showed that in the

sensitization phase, there was no increase in serum IgG2a concentrations in the negative control group. This could be occurred due to allergen sensitization caused Th0 cell differentiation shifted from Th1 and/or Treg to Th2. So that the process of IgG2a formation triggered by Th1 or Treg cells becomes very minimal or even no IgG2a is formed[25,26]. SAE immunotherapy was able to increase the serum concentration of IgG2a dose-dependently in an allergic mouse model. This indicates that SAE is an effective immunotherapy agent and dose-dependent based on increasing serum IgG2a concentrations. IgG production was often dependent on the induction of regulatory B cells. IgG2a in mice and IgG4 in humans, driven by Th1 or Treg cells induced by immunotherapy, act as blocking antibodies and positively correlate with clinical improvement in mice or allergic patients. As a fast-acting blocking antibody, IgG acts through the formation of IgG/FcγRIIb complexes on mast cells, resulting in downregulation of FcεRI receptor signaling and mast cell degranulation, sequestration of circulating allergens by induced IgG, and IgE internalization facilitated by IgG/FcγRIIb immune complex formation[23,27].

The induction of Treg cells and the immunity shifting from Th2 cells to Th1 cells are indicators of the success of allergic immunotherapy[27,28]. Regulatory T cells (Treg) play a critical role in regulating immune tolerance to allergen exposure by secreting anti-inflammatory mediators, one of which is IL-10[29]. There was no change in IL-10 mRNA expression in the negative control group compared to the normal group in this study. These results are similar to the results in serum IgG2a concentrations because the IL-10 expression pathway is the same as the IgG2a formation pathway, namely through Treg cells[26,30]. Increased expression of IL-10 mRNA was found in the immunotherapy group and was dose-dependent. This indicates that SAE is effective as an immunotherapy agent based on the increased expression of IL-10 mRNA in the ileal tissue of a gastrointestinal allergic mouse model and is dose-dependent. Increased expression of IL-10 mRNA, which is part of the Treg cell signaling pathway, correlates with decreased allergic response in a mouse model of gastrointestinal allergy[14].

CONCLUSION:

We have investigated the effectiveness of shrimp allergenic extract (SAE), developed in Indonesia, as an immunotherapy agent and its dose-dependent effect. The data demonstrate that SAE had excellent effects that could be seen from the decrease in systemic allergy symptom scores, the increase in IgG2a serum levels, and the increase in IL-10 mRNA expression in ileum tissues.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation

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