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Original Article

Sensitization with *Eustrongylides* sp. (Nematoda: Dioctophymatidae) antigens induce production of specific IgG and IgE in murine model

Sensibilização com antígenos de *Eustrongylides* sp. (Nematoda: Dioctophymatidae) induzindo a produção de IgG e IgE em modelo murino

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Abstract

Fish consumption plays an important role in human diet. *Hoplias malabaricus*, commonly known as traíra, is a freshwater fish widely appreciated in several Brazilian states and frequently infected by *Eustrongylides* sp. fourth-instar larvae (L4). The aim of the present study was to evaluate allergenic potential of *Eustrongylides* sp. L4 crude extract (CEE). BALB/c mice were immunized intraperitoneally (IP) by 10 µg CEE with 2 mg of aluminum hydroxide on days 0 and 35. Specific IgG and IgE antibody levels were determined after immunization and cellular immunity was evaluated by assessing intradermal reaction in ear pavilion. Epicutaneous sensitization was performed in dorsal region by antigen exposure using a Finn-type chamber containing 50 µg of CEE or saline solution, followed by evaluation of specific antibody levels. IP immunization resulted in a gradual increase in IgG antibody levels and transitory IgE production. Significant increase in ear thickness was observed in cellular hypersensitivity reaction. In case of antigen exposure by epicutaneous route, CEE was able to induce meaningfully increased levels of specific IgG and IgE antibodies as well as heightened cellular immunity. Both intraperitoneal immunization and epicutaneous contact with *Eustrongylides* sp. larval antigens were observed for first time to be capable of inducing immunological sensitization in mice.

Keywords: Nematode, allergenic potential, epicutaneous sensitization, intraperitoneal sensitization, murine model.

Resumo

Consumir peixe constitui papel importante na dieta humana. *Hoplias malabaricus*, comumente chamado de traíra, peixe de água doce largamente apreciado no Brasil, é frequentemente infectado com larvas de quarto estágio (L4) de *Eustrongylides* sp. O presente estudo objetivou avaliar o potencial alergênico do Extrato Bruto de L4 de *Eustrongylides* sp. (EBE). Camundongos BALB/c foram imunizados intraperitonealmente (IP) por 10 µg de EBE com 2 mg de hidróxido de alumínio nos dias 0 e 35. Após imunização, determinaram-se níveis específicos de anticorpos IgG e IgE e avaliou-se a imunidade celular pela reação intradérmica no pavilhão auricular. Realizou-se sensibilização epicutânea na região dorsal pela exposição ao antígeno, utilizando-se câmara tipo Finn, contendo 50 µg de CEE ou solução salina. Após exposições, foram avaliados níveis específicos de anticorpos. Na imunização

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via IP, houve aumento gradual nos níveis de anticorpos IgG e produção de IgE transitória. Foi observado aumento significativo na espessura do pavilhão auricular na reação de hipersensibilidade celular. Na exposição ao antígeno pela via epicutânea, o EBE induziu aumento significante nos níveis de anticorpos IgG e IgE específicos e induziu imunidade celular. Pela primeira vez, observou-se que a imunização intraperitoneal e contato epicutâneo com antígenos larvares de *Eustrongylides* sp. são capazes de induzir sensibilização imunológica em camundongos.

Palavras-chave: Nematoide, potencial alergênico, sensibilização epicutânea, sensibilização intraperitoneal, modelo murino.

Introduction

Studies of fish-related allergies have increased in recent years (Davis et al., 2020). Studies examining this phenomenon have shown that allergies are often not related to fish and seafood antigens themselves and may also occur by accidental ingestion of nematode parasite larvae of the family Anisakidae (Nieuwenhuizen et al., 2006).

Another nematode of zoonotic importance, and which has been commonly found parasitizing *Hoplias malabaricus* (Bloch, 1794), traíra, is *Eustrongylides* sp. Life cycles of *Eustrongylides* spp. include multiple hosts; piscivorous birds serve as the terminal host and oligochaetes and small predatory fish act as intermediate hosts (Moravec, 1998; Knoff et al., 2013). Species of the genus *Eustrongylides* Jägerskiöld, 1909 have been observed parasitizing fish in the United States (Choudhury et al., 2004; Mitchell et al., 2009; Bauer & Whipps, 2013; McAllister et al., 2016a, b; Muzzall & Hessenauer, 2018), Mexico (Salgado-Maldonado et al., 2004; Rosas-Valdez et al., 2007; Martínez-Aquino et al., 2012; Pinacho-Pinacho et al., 2014; García-López et al., 2016; Bautista-Hernández et al., 2019; Mosqueda-Cabrera et al., 2019), Argentina (Brugni & Viozzi, 1999, 2003; Guagliardo et al., 2019), Turkey (Aydoğdu et al., 2011; Çolak, 2013), Italy (Dezfuli et al., 2015; Branciari et al., 2016), Poland (Mierzejewska et al., 2012), Bulgaria (Nachev & Sures, 2009), Romania (Urdes et al., 2015), Iceland (Kristmundsson & Helgason, 2007), Serbia (Bjelić-Čabrilo et al., 2013; Djikanović et al., 2018), China (Moravec et al., 2003), Japan (Abe, 2011; Moravec & Nagasawa, 2018) and Australia (Chapman et al., 2006). In Brazil, *Eustrongylides* sp. larvae have been reported parasitizing *H. malabaricus* by several authors (Rego & Eiras, 1988; Rego & Vicente, 1988; Barros et al., 2007; Martins et al., 2009; Benigno et al., 2012; Meneguetti et al., 2013; Rodrigues et al., 2017; Kuraiem et al., 2020). *Hoplias malabaricus* is a predatory fish that feeds on a wide variety of other fish species and inhabits freshwater aquatic ecosystems in Central and South America (Godoy, 1975; Barros et al., 2007; Montenegro et al., 2013).

Human infections caused by the consumption of raw or undercooked fish that have been parasitized by fourth-instar larvae (L4) of *Eustrongylides* sp., leading to gastritis and intestinal perforation, have been reported in the United States and South Sudan (CDC, 1982; Gunby, 1982; Eberhard et al., 1989; Wittner et al., 1989; Deardorff & Overstreet, 1991; Narr et al., 1996; Mitchell et al., 2009; Knoff et al., 2013, Eberhard & Ruiz-Tiben, 2014).

Human exposure to anisakid nematode larvae has become a prominent public health issue because of its association with allergy symptoms. These symptoms range from localized reactions to generalized manifestations such as allergic rhinitis, conjunctivitis, asthma, urticaria, angioedema, allergic contact dermatitis and anaphylaxis (Armentia et al., 1998; Daschner et al., 2000; Nieuwenhuizen et al., 2006; Audicana & Kennedy, 2008; Uña-Gorospe et al., 2018).

Anisakis spp. are known to be capable of sensitizing the immune system, resulting in severe allergic and gastrointestinal reactions (Hochberg & Hamer, 2010; Cho et al., 2014; Morozińska-Gogol, 2019). Recent studies have demonstrated the potential of immunological sensitization in murine model by *Contracaecum multipapillatum* (Fontenelle et al., 2018) and *Hysterothylacium deardorffoverstreetorum* (Ribeiro et al., 2017) larvae.

Skin sensitization by food allergens have been reported (Perkin et al., 2020), and antigens applied by the epicutaneous route have been used as potent inducers of food allergies (Wang et al., 1996; Hsieh et al., 2003; Dunkin et al., 2011), suggesting that epicutaneous exposure plays an important role in the cause of atopic allergic dermatitis. For humans, approximately 80% of patients with atopic dermatitis (AD) have elevated serum levels of total and specific IgE to environmental and/or food allergens (Leung, 2000). Epicutaneous exposure to protein antigens has been reported as one of the important pathways for the development of AD (Santamaria Babi et al., 1995; Teraki et al., 2000). To date, there are no data about the allergenic potential of freshwater fish parasites, such as *Eustrongylides* sp. In this sense, the present study aimed to analyze the allergenic potential of *Eustrongylides* sp. larvae, collected from *H. malabaricus*, through sensitization to crude extract by intraperitoneal and epicutaneous routes in a murine model.

Materials and Methods

From April, 2016 to April, 2018, 45 specimens of *H. malabaricus* with total length of 13-46 cm (24.84 cm ± 7.52) and weight of 25-1,110 g (220.26 g ± 230.37) were collected by local fishermen from the municipality of Magé, in the state of Rio de Janeiro, Brazil (22º39'10" S; 43º02'26" W). The fish were transported in isothermal boxes with ice to the Universidade Federal Fluminense (UFF) in the municipality of Niterói, Rio de Janeiro, Brazil, where they were eviscerated and filleted. Parasitic nematodes were removed and observed under an Olympus BX-41 (Tokyo, Japan) brightfield microscope, with identification as *Eustrongylides* sp. being based on the descriptions of Measures (1988), Moravec (1998) and Kuraiem et al. (2020). All larvae used to prepare crude extract were collected and taxonomically identified by Kuraiem et al. (2020). Samples were washed repeatedly with 0.65% NaCl solution and then disintegrated in a Potter homogenizer (Thomas, Philadelphia, USA). Phenylmethylsulphonyl fluoride (PMSF) was then added followed by centrifugation at 10,000 rpm at 4 °C for 20 minutes. Protein quantification of the extracts was performed by the method of Lowry et al. (1951), using bovine serum albumin (BSA) 1 mg per ml as the standard. For the experiment, 8-10-week-old BALB/c mice were raised and kept in the local bioterium in the Laboratory Animal Nucleus, UFF. This study was approved by the Animal Research Ethics Committee of the Universidade Federal Fluminense (UFF) Centre for Laboratory Animals (1046/ 2018). Immunization with crude extract of *Eustrongylides* sp. (CEE) involved intraperitoneal injections (IP) with 10 µg CEE associated with 2 mg of aluminum hydroxide solution on the initial day of the experiment (day 0 or primary immunization) and on day 35 for an experimental group consisting of five BALB/c mice. Blood samples of the mice were collected on days 0, 14, 21, 35, 42, 49, 63, 77, 84 and 91 to determine the levels of specific antibodies in serum using the enzyme-linked immunosorbent assay (ELISA). Sensitization by the epicutaneous route consisted of two experimental groups of five BALB/c mice. The animals were anaesthetized and depilated in the dorsal region on days 0, 7 and 25, for the adhesion of a Finn-type chamber (Finn Chambers®, Epitest Ltd Oy, Tuusula, Finland). The control group was exposed to a 0.9% NaCl solution and the immunized group was exposed to 50 µg CEE. There were three application steps, which took four days each to complete. Blood samples were collected to obtain serum on days 0, 28 and 43 to evaluate specific antibody levels by ELISA. The presence of anti-*Eustrongylides* sp. IgG and IgE isotypes in the sera of mice was determined by ELISA (Verícimo et al., 2015). Briefly, in Nunc MaxiSorp™ flat-bottom plates (Nunc, Roskilde, Denmark) 50 µl of CEE antigens (containing 20 µg protein/ml) were placed in a 0.05 M carbonate/bicarbonate buffer, pH 9.6. The sera were serially diluted from 1 to 40 in PBS-G (phosphate-buffered saline gelatine, pH 7.2) at base 3 and incubated for 2 h at 37 °C. After washing with phosphate-buffered saline-Tween (PBS-T), peroxidase-conjugated antibodies anti-total IgG (L and H) (1: 10,000) (rabbit anti-mouse IgG, whole molecule, Sigma-Aldrich, St. Louis, Missouri, USA) were used for 1 h at 37ºC. The IgE analysis was performed in two steps. First an anti-IgE (ε chain) (1:300) (rat anti-mouse IgE, Invitrogen, Carlsbad, California, USA) for 1 h at 37ºC, and then, after washing with PBS-T, peroxidase-conjugated antibodies anti-rat IgG (1:10,000) (HRP-goat anti-rat IgG Life Technologies, Carlsbad, California, USA) for 1h at 37ºC. After another washing with PBS-T, the reaction with the substrate and chromogen was performed similarly for IgG and IgE by adding 50 µl per well of o-phenylenediamine and H_2O_2 (diluted in citrate phosphate buffer, pH 5.0) solution (OPD). The enzymatic reaction was stopped with the addition of 4N sulphuric acid. Optical density (OD) reading was performed on a microplate reader (Anthos 2010, Krefeld, Germany) at a wavelength of 492 nm. The analysis of the results was performed by comparing the sum of the OD of each serum. The results are reported as arbitrary units of ELISA corresponding to the value of the area under the dilution curve of each serum. For the evaluation of cellular immunity on day 91 (end of the intraperitoneal experiment) and day 43 (end of the epicutaneous experiment), the animals received an intradermal injection of 20 µl of CEE in the left ear pavilion and 20 µl of 0.90% NaCl solution in the right ear pavilion as a control. Ear pavilion thickness was measured before inoculation and at 24, 48 and 72 h thereafter, with a nº 7301 micrometre (Mitutoyo Sul Americana, Rio de Janeiro, Brazil). Data were analysed by repeated measures one-way ANOVA followed with multiple comparisons by post hoc Tukey's, using the program GraphPadPrism-version 6 for Windows XP (GraphPad Software, San Diego, CA, USA, www.graphpad.com Copyright 1992-1998). Values were considered to be significant if $p < 0.05$.

Results

Results of the evaluation of the intensity of the immunological response induced by intraperitoneal route are shown in Figure 1. After primary immunization with CEE, a statistically significant increase in specific IgG antibodies levels (Figure 1A) against CEE occurred on days 14 and 42 when compared to day 0 (control prior to immunization) ($p < 0.01$ for day 14 through 35 and < 0.001 for days 42 through 91). After the second immunization, performed on day 35, an increase in the levels of these antibodies was observed, which remained at the same level until day 91

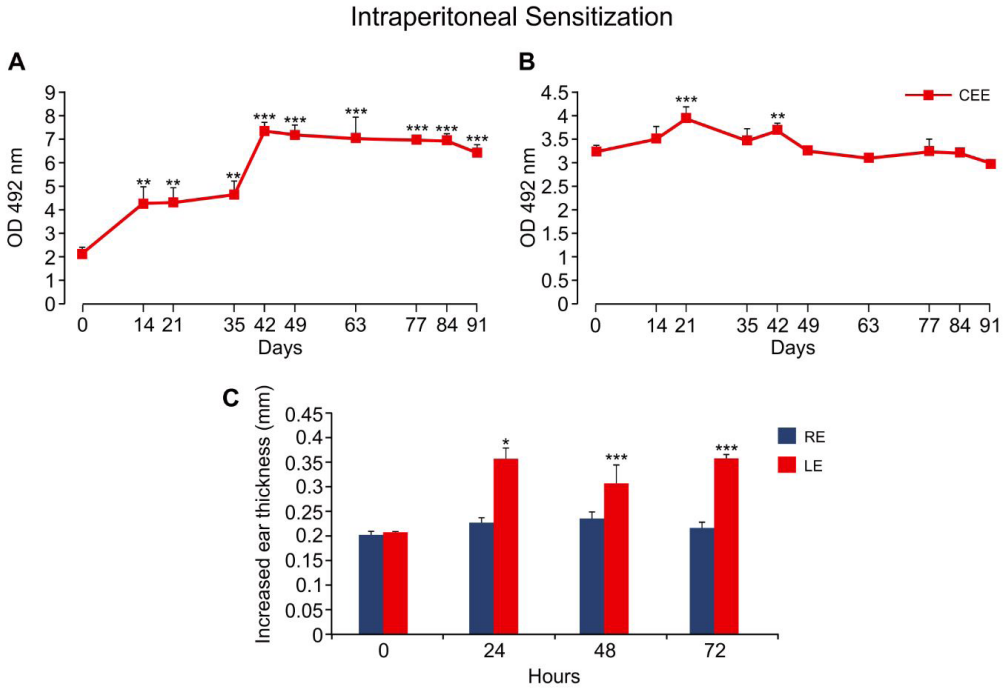

Figure 1. Intraperitoneal sensitization. Immunogenicity of the crude extract of *Eustrongylides* sp. (CEE). IgG and IgE levels and delayed hypersensitivity reaction. Specific IgG kinetics (A) and specific IgE kinetics (B) for a group of five mice that received intraperitoneal injections of 2 mg of 10 µg of CEE associated with 2 mg Al $(OH)^3$ on days 0 and 35. The values indicate the averages of the sum of the optical densities (OD) ± the standard deviation (SD) of the group in serum samples at days 0, 14, 21, 35, 42, 49, 63, 77, 84 and 91; (C) Cell hypersensitivity reaction after challenge with 20 µl CEE in the left ear pavilion (LE). The result represents the increase in ear pavilion thickness in millimeters and ± SD for the LE and the right ear (RE) pavilion as control, at hours 0, 24, 48 and 72. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, compared to the serum sample taken prior to immunization (Figure 1A, B) and to hour zero (Figure 1C).

($p < 0.001$). IgE production levels exhibited a transitory elevation at day 21 after the first immunization ($p < 0.001$), and a modest and transitory elevation after the second immunization at day 42 ($p < 0.01$) (Figure 1B). Evaluation of the cell hypersensitivity reaction is presented in Figure 1C. Cellular immunity revealed a significant increase in ear pavilion thickness when challenged with CEE at hours 24 ($p < 0.05$), 48 and 72 ($p < 0.001$) compared to hour zero.

The intensity of the immunological response induced by the epicutaneous route is represented in Figure 2. After sensitization on days 28 and 43, a significant increase in specific IgG antibodies against CEE occurred, when compared to the day 0 of the same group ($p < 0.05$ for day 28 and < 0.01 for day 43) (Figure 2A). The CEE group presented IgE levels with a transitory elevation at day 28 compared to day 0 of the same group ($p < 0.001$) (Figure 2B). Evaluation of the cell hypersensitivity reaction is presented in Figure 2C. The animals showed a statistically significant increase in left ear pavilion thickness from the initial time until 24 h had elapsed ($p < 0.05$) with a growth of inflammation at hour 48 and maintenance of the same statistical level of inflammation until hour 72 ($p < 0.001$) compared to right ear pavilion and hour zero.

Discussion

The intraperitoneal administration of CEE associated with the immune adjuvant aluminum hydroxide induced the production of IgG and IgE antibodies. This finding is in agreement with experimental immunization for the purpose of inducing high levels of IgE antibodies. Immune-activating immunization requires the combination of the antigen and adjuvants, such as cholera toxin, pertussis toxin or aluminum hydroxide, as reported by Brewer et al. (1999) and Li et al. (2000). In general adjuvants are thought to stimulate certain cell populations involved in antibody responses, and the target of adjuvant effect may vary from one to the other. In this study, was demonstrated that 10 ug the crude extrat derived from fourth-instar larvae from *Eustrongylides* sp. associated to aluminum hydroxide stimulate an immune response with secretion of high levels of circulating IgG and IgE .

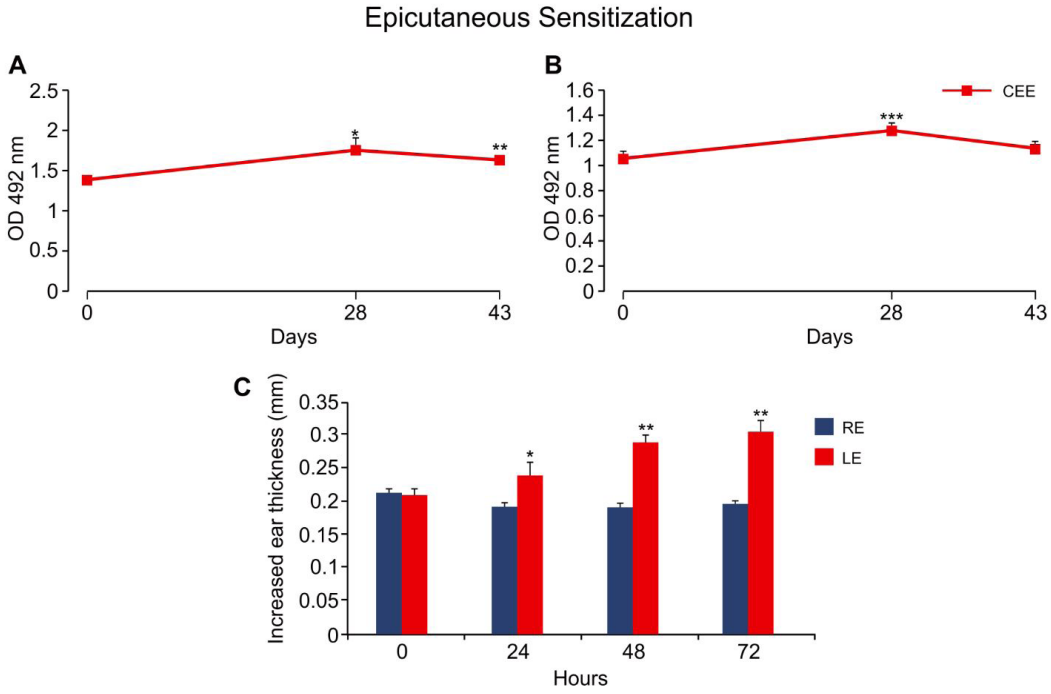

Figure 2. Epicutaneous sensitization. IgG and IgE levels and delayed hypersensitivity reaction. Specific IgG kinetics (A) and specific IgE kinetics (B) for a group of five mice sensitized with 50 µg crude extract from *Eustrongylides* sp. (CEE) via the epicutaneous route on days 0, 7 and 25, Group CEE. The values represent the mean optical density (OD) ± standard deviation of the group in serum samples at days 0, 28 and 43; (C) Cell hypersensitivity reaction after challenge with 20 µl CEE in the left ear pavilion (LE). The result represents the increase in ear pavilion thickness in millimeters ± SD for the LE and right ear (RE) pavilion as control at hours 0, 24, 48 and 72. *p < 0.05, **p < 0.01 and ***p < 0.001, compared to the serum sample taken prior to immunization (Figure 2A, B) and to hour zero (Figure 2C).

The result of present study is in agreement with Fontenelle et al. (2018) that challenged mice by intraperitoneal and epicutaneous routes using a crude extract of *C. multipapillatum* (CECM), an anisakid nematode collected from *H. malabaricus*. These authors reported a significant production of specific IgG and IgE antibodies, which corresponds with the results of the present study by both routes, but they found higher IgG and lower IgE levels, although they used a different parasite and a higher dose of crude extract (50 µg CECM) in their protocol.

Immunization via the intraperitoneal route performed in the present study differs from the experimental protocol developed by Baeza et al. (2005). Using their protocol, these authors were only able to sensitize animals after multiple intraperitoneal inoculations of 100 µg of *A. simplex* antigens associated with two adjuvants, aluminum hydroxide and pertussis toxin. The present study demonstrated that utilizing even a small concentration of somatic antigens of *Eustrongylides* sp. larvae associated with aluminum hydroxide was sufficient to induce specific IgG and IgE antibodies, with a significant increase in the thickness of the ear pavilion of the immunized animals. The present results suggest an immunization with a mixed Th1 / Th2 response, as was suggested by Baeza et al. (2005).

In the present study, a lower concentration of the somatic parasite antigen CEE was effective in initiating IgE production. In comparing the extracts of the two studies (Baeza et al., 2005; Fontenelle et al., 2018), it is important to note that these antigens were extracted from nematode larvae of different families, which could be a reason for the differences in immunological response.

In the present study, the late hypersensitivity reaction was verified by measuring an increase in ear pavilion thickness in the first 24 hours, which can be attributed to the activity of specific antibodies that form immune complexes at the site of the antigen inoculation and alter the vascular permeability and induce the formation of oedema. Also observed in the present study was the maintenance of the thickness of the dermis at 48 and 72 h, which is compatible with mononuclear cell infiltration at the site of inoculation, as reported by Nieuwenhuizen et al. (2006), for reactions of allergic contact dermatitis in humans.

The present study observed low induction of IgE production. This result differs from observations reported by Hsieh et al. (2003), which showed a high induction of IgE production. This difference could come from the longer parasite antigen exposure time (seven days) than that of the present study (four days).

The results reported here for *Eustrongylides* sp. and the epicutaneous route show that both triggered an immune sensitization. The possibility, and pathway, of cutaneous hypersensitivity in humans by this nematode should be elucidated in future studies, since this has direct implications for the possible induction of allergy in personnel involved in the preparation of fish for consumption, whether they are an industrial worker, fishmonger or consumer, as suggested by Fontenelle et al. (2018), emphasizing its zoonotic importance.

Both intraperitoneal immunization and epicutaneous contact with *Eustrongylides* sp. larval antigens were observed for first time to be capable of inducing immunological sensitization in mice. The results obtained in the present study suggest that sensitization of mice with *Eustrongylides* sp. antigens is capable of inducing sensitization of the immune system. This, in turn, results in considerable antibody production and significant cellular immunity, and further suggests that allergen exposure via the epicutaneous route may be a natural pathway for food allergy sensitization, which can aid in better understanding the pathogenesis of this allergic disease.

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