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RESEARCH NOTE

Oral inoculation of live or dead third-stage larvae of *Anisakis simplex* in rats suggests that only live larvae induce production of antibody specific to *A. simplex*

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Abstract

Live *Anisakis simplex* third-stage larvae (L3) penetrate gastrointestinal mucosa after they are ingested in raw or undercooked seafood, thereafter causing gastrointestinal manifestations and allergic manifestations such as urticaria and anaphylaxis. These allergic reactions are mediated by specific IgE to L3 allergens, especially excretory–secretory (ES) allergens. Recent evidences suggest that only live larvae can cause allergic reactions, although cases attributable to ingestion of cooked, frozen seafood have been reported. Therefore the risk of *Anisakis*-associated hypersensitivity by ingestion of properly cooked and frozen fish remains controversial. No prior report describes the kinetics of antibody production in experimental animals after oral inoculation with dead L3. This study used ELISA to assess antibody production in rats inoculated orally with dead L3. Positive absorbance value in IgG, IgM, and IgE specific to ES antigen from L3 were found in rats inoculated with live L3 but not with dead L3 (frozen, heated, cut, or homogenized). At one week post re-inoculation with live or frozen L3 to the initially sensitized rats, the absorbance value of the specific IgM and IgE to ES antigen elevated quickly and highly in rats that had been re-inoculated with live L3, but they decreased slightly or did not change in rats inoculated with frozen L3. These results suggest that only ingestion of live L3 can produce the specific antibody and induce initial and secondary sensitizations to L3.

Keywords

Anisakis simplex, ELISA, excretory–secretory antigen, IgE, allergy, rat, experimental infection

Marine mammals such as seals, whales, and dolphins are the definitive hosts of *Anisakis simplex*, a parasitic nematode infecting fish and mammals. The intermediate or paratenic hosts include species of marine fish such as mackerel, codfish, sardine, anchovy, salmon, and squid. Human infection, known as anisakiasis, occurs through the ingestion of raw or undercooked seafood. In fact, live *A. simplex* third-stage larvae (L3) are well known to cause allergic manifestations such as urticaria and anaphylaxis as well as gastrointestinal manifestations after ingestion of presumably parasitized fish or their products. These allergic reactions are thought to be mediated by IgE that is specific to *A. simplex* allergens (Audicana and Kennedy 2008). Recent evidence suggests that only the ingestion of live larvae, which attach themselves to the human gastric mucosa, can cause these reactions (Sastre *et al.* 2000; Alonso-Gómez *et al.* 2004; Baeza *et al.* 2004). However, cases of *Anisakis*-associated hypersensitivity attributable to ingestion of cooked, canned, and frozen seafood have also been suggested in countries where *Anisakis* are endemic (Audicana *et al.* 1997; Kameyama *et al.* 2006; Audicana and Kennedy 2008). Therefore, the risk of *Anisakis*-associated allergic reaction by ingestion of properly cooked and frozen fish remains controversial (Daschner *et al.* 2012; Pravettoni *et al.* 2012). Oral inoculation of rats with live L3 is known to be a useful experimental model for analyse of allergic responses in anisakiasis (Cho *et al.* 2005). To date, seven *A. simplex* excretory–secretory (ES) allergens that were found to be thermostable or resistant to pepsin digestion have been reported.

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The patients, including allergic patients, develop specific IgE to these allergens (Baeza *et al.* 2004; Audicana and Kennedy 2008; Pravettoni *et al.* 2012). Oral challenges with lyophilized L3 or excretory–secretory extracts from L3 to sensitized patients who experienced several *Anisakis*-associated allergic reactions (urticaria, angioedema, anaphylaxis, or abdominal pain) after eating marinated, grilled or raw fish did not induce allergic manifestations (Baeza *et al.* 2004; Sastre *et al.* 2000). The possibility exists that the condition of dead L3 inoculated as an allergen influenced those findings because of a potential loss of allergenicity during the lyophilisation process and exposure to gastric acid or pepsin in the stomach. Therefore, to evaluate the allergenicity of ingested dead L3, it is appropriate and practical to use L3 that have been inactivated using various methods. No report in the literature has described the kinetics of antibody production in experimental animals that have been orally inoculated with dead L3, aside from an experiment for inoculation of live L3 cut in half or thirds to guinea pigs (Asami and Inoshita 1967). The present study used indirect enzyme linked immunosorbent assay (ELISA) to examine the possibility of production of IgE antibody that is specific to ES antigen in rats inoculated orally with dead L3 prepared by freezing, heating, cutting, and homogenization.

Anisakis simplex L3 were collected from viscera of chub mackerel (*Scomber japonicus*) and were washed with phosphate buffered saline (PBS) to remove fish contaminants. To obtain ES antigen, six-well microplates with 20 viable L3 in 4 ml Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen Japan K.K.) containing D-glucose (0.1 g/ml), antibiotic–antimycotic and kanamycin sulphate liquid (Invitrogen Japan K.K.) in each well were cultured at 37°C in a 5% CO_2 incubator for 5 days. The supernatant was concentrated using a Vivaspin 4 (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and was dialyzed against PBS using Slide-A-Lyzer Dialysis Cassettes (Thermo Fisher Scientific Inc., Rockford, IL, USA). Protein concentrations of ES antigen were estimated using the Quick Start Bradford Protein Assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). They were stored (20 µg/ml) at –20°C until use. The other live L3 were provided for oral inoculation to rats, as described below.

ELISA plates (Micro Test III Flexible Assay Plate; Becton Dickinson and Co., Franklin Lakes, NJ, USA) were coated with 100 µl of 0.1 µg/ml of ES antigen in carbonate–bicarbonate buffer (pH 9.6) overnight at 4°C. The plates were then washed twice with PBS containing 0.05% Tween 20 (PBST) and were blocked with 200 µl per well of Starting Block T20 (PBS) Blocking Buffer (Thermo Scientific, Inc., IL, USA) for 1 h at 37°C. After washing two times with PBST, the plates were incubated for 1 h at 37°C with each serum sample diluted to 1:400 in PBST. Plates were then washed three times with PBST and were incubated for 1 h at 37°C with horseradish peroxidase labelled goat antibody anti-rat-IgG (American Qualex Inc. (AQ), San Clemente, CA, USA) diluted 1:20,000 in PBST or with horseradish peroxidase labelled goat antibody anti-rat IgM (AQ) diluted 1:80,000 in PBST. Then the plates were washed five times with PBST. SureBlue Reserve TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories (KPL) Inc., Gaithersburg, MD, USA) was used as substrate. The reaction was stopped by adding TMB Stop Solution (KPL). The absorbance value at 450 nm was measured (Labsystems Multiskan MS; Thermo Scientific, Inc.). For the detection of specific IgE, ELISA plates were coated with 100 µl of 0.5 µg/ml of ES antigen and incubated for 2 h at 37°C with each serum diluted to 1:200. The plates were incubated for 1 h at 37°C with mouse monoclonal antibody anti-Rat-IgE (MARE-1; American Research Products, Inc., Waltham, MA, USA) diluted 1:100. Then plates were incubated for 1 h at 37°C with horseradish peroxidase labelled goat antibody anti-mouse-IgG (AQ) diluted to 1:10,000.

Eight-week-old male Wistar rats (Japan SLC Inc., Hamamatsu, Japan) were kept in an animal room of the Osaka City Institute of Public Health and Environmental Sciences, according to the guidelines of the Experimental Animal Committee of this institution for laboratory animals. All animals were maintained under a 12 h light–dark cycle with free access to water and standard laboratory food. Five experimental groups were orally inoculated with live or dead L3 placed on the pharynx or directly into the stomach. Rats in groups LL3, FL3, HL3, CL3, and HML3 were inoculated respectively with 10 live L3, 10 frozen L3 (–80°C for 1 h), 10 heat-treated L3 (60°C for 1 h), 10 L3 cut in half, and 10 homogenized L3, respectively. Homogenization of L3 was done as follows: 10 L3 were ruptured using microtube pestles (Scientific Specialties Inc., Lodi, CA, USA) and sonicated on ice at 100 W for five periods of 30 s using an ultrasonic homogenizer (US-300T; Nihon Seiki Kaisha, Ltd., Tokyo, Japan). Each group included five rats. All rats were bled under ether anaesthesia from the tail once every week until 4 weeks post live or dead L3 inoculation. The sera were stored at –20°C until use. Sera from normal rats before oral inoculation of live or dead L3 were used as negative control. The averages of absorbance values from two wells for every serum sample in each group are presented as line graphs (Fig. 1). The absorbance value exceeding the mean absorbance value of the negative control serum plus three standard deviations (SD) was considered positive in a specific antibody.

According to a previous study (Cho *et al.* 2005) the absorbance value of the specific antibodies, especially IgM and IgE, in primary sensitized rats by inoculation with live L3 were found quickly and were highly elevated after re-inoculation with live L3. In this study, the frozen L3 were inoculated to the primary sensitized rats to confirm the possibility of inducing elevation of specific antibody by ingestion of dead L3. The rats that had been primarily inoculated orally with live L3 were re-inoculated with 10 live (Fig. 2, R1-R3) or 10 frozen L3 (Fig. 2, R4-R6) after 8 weeks post primary inoculation of live L3. They were bled from the tail once a week until 13 weeks post primary inoculation. The kinetics of the absorbance value of specific antibodies to ES antigen was measured following the method described above. The average

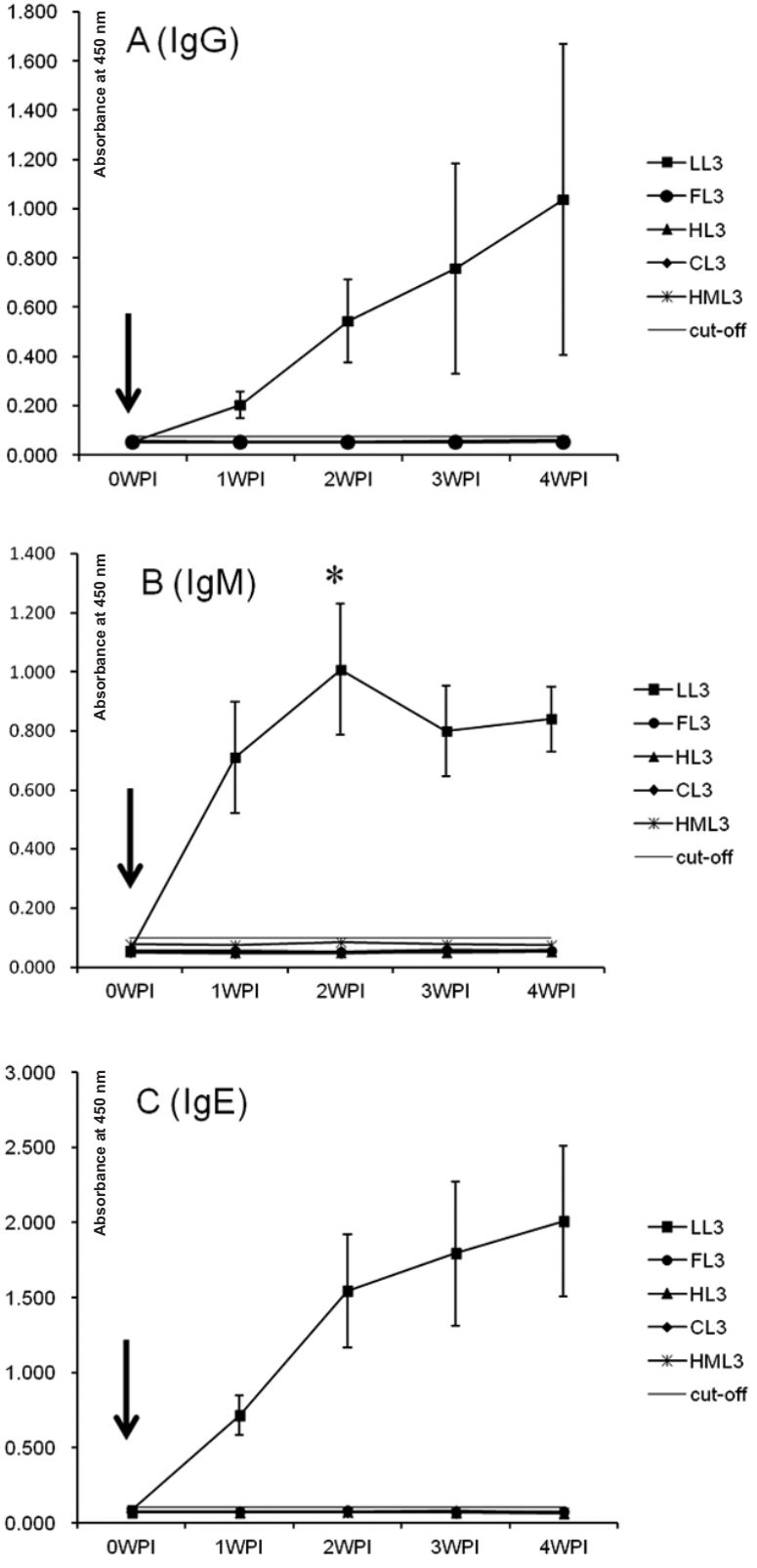

Fig. 1. Kinetics of specific antibody (A, IgG; B, IgM; C, IgE) production against ES antigen from L3 in rats inoculated with live L3 (LL3), frozen L3 (FL3), heated L3 (HL3), cut L3 (CL3), and homogenized L3 (HML3) during the experimental period. SD is shown as bars in the group LL3. WPI denotes the weeks post inoculation. Closed arrows denote inoculation with live L3. The mean absorbance value after a 2 week period in IgM was significantly higher than that after 1 or 3 weeks (*, $p<0.01$)

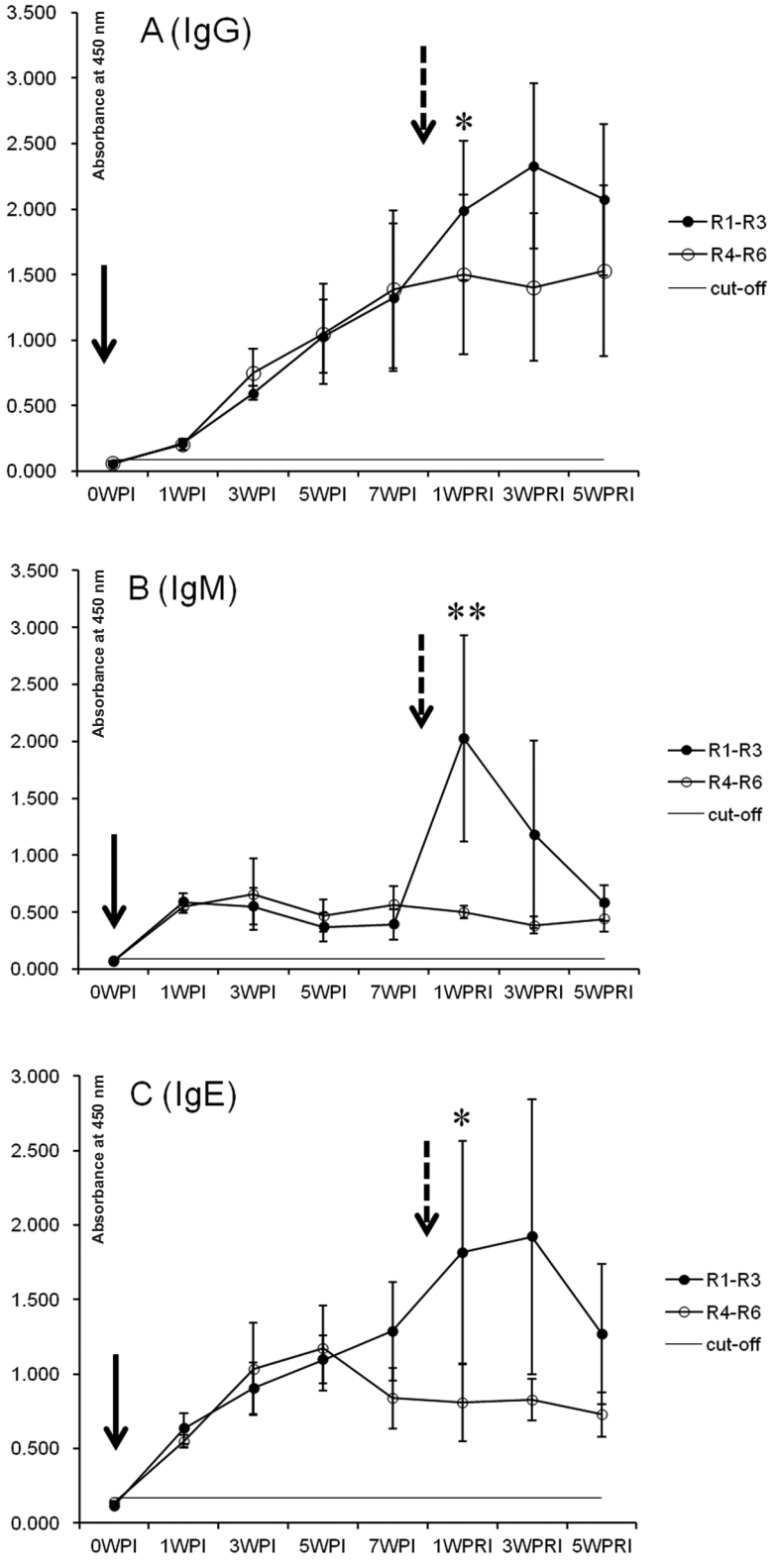

Fig. 2. Kinetics of specific antibody (A, IgG; B, IgM; C, IgE) production against ES antigen from L3 in sensitized rats after re-inoculation with live L3 (R1–R3) or frozen L3 (R4–R6) during the experimental period. SD is shown as bars in both groups. WPRI denotes the weeks post re-inoculation. Solid and dashed arrows respectively denote primary inoculation with live L3 and secondary inoculation with live or frozen L3. Significant differences in the mean absorbance values of IgG, IgM, and IgE between 7WPI and 1WPRI were found in one group (R1-R3), but none was observed in the other group (R4-R6). *p<0.05, **p<0.01

of absorbance values from two wells for every serum sample in each group is presented in line graphs (Fig. 2). The cut-off value setting is the same as that described above. For statistical analyses, T.TEST, one-way analysis of variance (ANOVA) or Tukey test was performed using the Statcel 3 software (The Publisher OMS Ltd., Tokyo, Japan). Probability *(p)* values less than 0.05 were inferred as significant.

The absorbance values of specific antibodies in rats inoculated with live L3 were elevated at one week after inoculation (Fig. 1, group LL3). IgG (Fig. 1A) and IgE (Fig. 1C) antibodies elevated gradually during experimental periods, although IgM levels peaked after a 2 week period and subsequently declined (Fig. 1B). The kinetics of specific antibodies observed in this study during 1–4 weeks after live L3 inoculation was almost identical to that of the previous study using rats inoculated with 20 live L3 (Cho *et al.* 2005). In contrast, the absorbance values of specific antibodies in rats inoculated with dead L3 were lower than those of the cut-off values during the experimental periods, irrespective of the methods used for inactivating L3 (Fig. 1, groups HL3, FL3, CL3, and HML3). An earlier report described that live L3 that have been cut in half or thirds retain their infectivity to some degree and invade into the submucosal tissue of the stomach after oral administration to guinea pigs (Asami and Inoshita 1967). However, the possibility for inducing the production of specific antibody by the ingestion of cut L3 remains unclear. Judging from the data obtained in this study, specific antibody production might not be induced by the ingestion of cut L3 in rats, although we did not confirm the invasion into the submucosal tissue of the gastrointestinal tract by cut L3 in rats. However, in the primary-sensitized rats, the absorbance values of the specific antibodies were elevated quickly and highly in rats (Fig. 2, R1–R3) that were re-inoculated with live L3. In the other rats re-inoculated with frozen L3 (R4–R6), the absorbance values of the specific antibodies IgG, IgM and IgE reached a plateau or decreased gradually (Fig. 2). This result supports the previous finding (Alonso *et al*. 1999; Alonso-Gomez *et al.* 2004) that the ingestion of frozen fish is safe in anisakiasis. Combining the data presented in Figs. 1 and 2, we were able to demonstrate that ingestion of live L3 is necessary to produce the specific antibody and to induce first and secondary sensitizations to L3. Gastrointestinal lesions together with altered gastrointestinal permeability might account for the low quantities of allergens because of contact with submucosal mast cells, producing a prolonged or chronic *Anisakis*-associated urticarial reaction. Further study using the experimental animal model with gastrointestinal lesions must be conducted to confirm the allergenicity of ingested dead L3.

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References

Alonso A., Moreno-Ancillo A., Daschner A., Lopez-Serrano M.C. 1999. Dietary assessment in five cases of allergic reactions due to gastroallergic anisakiasis. *Allergy*, 54, 517–520.

Alonso-Gómez A., Moreno-Ancillo A., López-Serrano M.C., Suarez-de-Parga J.M., Daschner A., Caballero M.T., Barranco P., Cabañas R. 2004. *Anisakis simplex* only provokes allergic symptoms when the worm parasitizes the gastrointestinal tract. *Parasitology Research*, 93, 378–384. DOI: 10.1007/s00436-004-1085-9.

Asami K., Inoshita Y. 1967. Experimental anisakiasis in guinea pigs: Factors influencing infection of larvae in the host. *Japanese Journal of Parasitology*, 16, 415–422.

Audicana L., Audicana M.T., De Corres L.F., Kennedy M.W. 1997. Cooking and freezing might not protect against allergenic reactions to ingested *Anisakis simplex* antigens in humans. *Veterinary Record*, 140, 235.

Audicana M.T., Kennedy M.W. 2008. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. *Clinical Microbiology Review*, 21, 360–379. DOI: 10.1128/CMR.00012-07.

Baeza M.L., Rodríguez A., Matheu V., Rubio M., Tornero P., De Barrio M., Herrero T., Santaolalla M., Zubeldia J.M. 2004. Characterization of allergens secreted by *Anisakis simplex* parasite: clinical relevance in comparison with somatic allergens. *Clinical and Experimental Allergy*, 34, 296–302.

Cho T.H., Park H.Y., Cho S., Sohn J., Yoon Y.W., Cho J.E., Cho S.W. 2005. The time course of biological and immunological allergy states induced by *Anisakis simplex* larvae in rats. *Clinical and Experimental Immunology*, 143, 203–208. DOI: 10.111/j.1365-2249.2005.02981.x.

Daschner A., Cuellar C., Rodero M. 2012. The *Anisakis* allergy debate: does an evolutionary approach help? *Trends in Parasitology*, 28, 9–15. DOI: 10.1016/j.pt.2011.10.001.

Kameyama R., Yagami A., Yamakita T., Nakagawa M., Nagase K., Ichikawa H., Matsunaga K. 2006. A case of immediate allergy to *Anisakis* following saury intake. *Japanese Journal of Allergology*, 55, 1429–1432. (in Japanese with English summary)

Pravettoni V., Primavesi L., Piantanida M. 2012. *Anisakis simplex*: current knowledge. *European Annals of Allergy and Clinical Immunology*, 44, 150–156.

Sastre J., Lluch-Bernal M., Quirce S., Arrieta I., Lahoz C., Del Amo A., Fernández-Caldas E., Marañón F. 2000. A double-blind, placebo-controlled oral challenge study with lyophilized larvae and antigen of the fish parasite, *Anisakis simplex*. *Allergy*, 55, 560–564.

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