

A *Kudoa septempunctata* antigen induces production of IgE in BALB/c mice

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Abstract *Kudoa septempunctata*, a myxosporean parasite, is the causative agent of a foodborne illness associated with consumption of raw *Paralichthys olivaceus* (olive flounder). Because the lag phase of this illness is short (from 1 to 12 h), it is possible that an allergic response is relevant to this illness. To test whether a *K. septempunctata* antigen is the possible allergen, we injected a myxospore extract into BALB/c mice and measured IgE levels in serum. When the mice were injected with the myxospore extract, the total serum IgE concentration increased significantly after the second immunization as compared to the negative control. After the third immunization, total IgE concentration in the immunized mice reached 26.5 ng/ml and was almost equivalent to that of egg albumin-injected mice. Western blot analysis revealed that IgE antibodies—in serum samples that were collected from myxospore extract-injected mice—bound to at least two *Kudoa* proteins with molecular weight between 28 and 36 kDa. These results suggested that a *K. septempunctata* antigen is the allergen. Further studies are needed to clarify the contribution of allergy to the foodborne illness caused by *K. septempunctata*.

Keywords *Kudoa* · Allergy · Foodborne illness · Fish

Introduction

Kudoa septempunctata, a myxosporean parasite, is considered the causative agent of a foodborne illness associated with the consumption of *Paralichthys olivaceus* (olive flounder) (Kawai et al. 2012). Myxospores of *K. septempunctata* reside in the trunk muscle of *P. olivaceus*, and the consumption of contaminated *P. olivaceus* meat leads to the foodborne illness (Kawai et al. 2012; Ohnishi et al. 2013). The symptoms include transient but severe diarrhea and emesis, but the patients usually recover within 24 h. The short lag phase of 1–12 h is one of the characteristics of this disease. At present, *K. septempunctata* is subject to the Food Sanitation Act in Japan. Therefore, the sale of *P. olivaceus* meat containing *K. septempunctata* myxospores is prohibited at fish shops or restaurants in Japan. Previously, many incidents had been caused by consumption of *P. olivaceus* raised on Japanese farms (Food Safety Commission of Japan 2015). Because all Japanese farms are land-based, it is believed that the life cycle of *K. septempunctata* is not maintained on these farms. Therefore, it is important that uninfected olive flounder fry that are checked by PCR are introduced to the farm with sterilized or filtered seawater used for cultivation. Japanese farms are now *K. septempunctata*-free because of these measures. Nonetheless, the incidents caused by imported *P. olivaceus* are still a substantial problem in Japan (Food Safety Commission of Japan 2015).

Myxospores that are ingested by humans release sporoplasm in the intestine, and the sporoplasm penetrates the human intestinal surface monolayer of cells, and this penetration contributes to diarrhea (Ohnishi et al. 2013). Because the temperature and osmotic pressure in the human intestine are not appropriate for residence of the sporoplasm, the latter will die within 72 h after causing symptoms (Ohnishi et al. 2016). It is believed that the quick resolution of symptoms is

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due to the inability of the sporoplasm to adapt to the conditions of the human intestine (Ohnishi et al. 2016).

It has been reported that the injection of *Kudoa* spp. isolated from hake induces production of IgG and IgE antibodies in mice (Martinez de Velasco et al. 2002). Because IgE binds to an allergen and stimulates mast cells to release inflammatory mediators such as histamine and prostaglandins, IgE is responsible for the induction of an allergic response (Burks et al. 2012). Some patients with gastroallergic and/or allergic symptoms related to fish ingestion test positive in the skin prick test for *Kudoa* sp. antigens (Martinez de Velasco et al. 2008). These reports indicate that an allergic reaction is one of the contributing factors of the *Kudoa* foodborne illness. Although it has been shown that a history of allergy is not related to the incidence of this foodborne illness (Yahata et al. 2015), the reports by Martinez et al. motivated us to study the involvement of allergy in the foodborne illness caused by *K. septempunctata*. In this study, we evaluated the possibility that the gastrointestinal symptoms caused by *K. septempunctata* are associated with an allergic response. Given that the diagnosis of food allergy is made by the induction of allergen-specific IgE in the serum of a patient, we first studied the production of IgE in mice that were injected with *K. septempunctata*.

Materials and methods

Preparation of the *K. septempunctata* antigens

P. olivaceus specimens were purchased at a local market in Japan. The infection of *P. olivaceus* fillets with *K. septempunctata* was histologically inspected, and *K. septempunctata* myxospores were isolated and purified as described previously (Kawai et al. 2012). Myxospores were resuspended in PBS (Thermo Fisher Scientific Inc., USA) at the concentration of 10^7 myxospores/ml and stored at -80°C before use. After thawing, the suspension of myxospores was sonicated by 20 pulses of 30 s with a Sonifier 200 (Branson Ultrasonics, Danbury, USA) set to 50% power output on ice. Because myxospores contain two sporoplasms, this myxospore extract comprises both myxospore-derived and sporoplasm-derived antigens. The myxospore extract was mixed with an equal volume of an aluminum hydroxide gel containing 19.8 mg/ml aluminum hydroxide (Wako Pure Chemical Industries, Ltd., Japan). Fifteen SPF BALB/c mice (4 weeks old; Japan SLC, Inc., Japan) were injected with this mixture (500 μl /mouse) intraperitoneally. After 10 days, the mice were injected with the myxospore extract (500 μl /mouse) without the aluminum hydroxide gel subcutaneously. Another 10 days later, the mice were injected with the myxospore extract (500 μl /mouse) without the aluminum hydroxide gel subcutaneously again. As a positive control, egg

albumin (8 mg/mouse for each immunization) was mixed with an equal volume of the aluminum hydroxide gel. Three mice were injected with the mixture of egg albumin with the aluminum hydroxide gel (500 μl /mouse) intraperitoneally, followed by two injections of egg albumin (500 μl /mouse) without the aluminum hydroxide subcutaneously at 10-day intervals. As a negative control, PBS was mixed with an equal volume of the aluminum hydroxide gel, and three mice were injected with this gel mixture intraperitoneally, followed by two injections of PBS without the aluminum hydroxide gel subcutaneously at 10-day intervals. Blood was sampled via tail vein prior to each immunization and 10 days after the third immunization under isoflurane anesthesia. Blood samples were centrifuged to prepare serum. The total IgE concentration was measured by a sandwich enzyme-linked immunosorbent assay (ELISA; Morinaga Institute of Biological Science, Inc., Japan). Before the assay, the serum samples were diluted with the dilution buffer supplied by the assay manufacturer. The results were reported as mean $\pm$ standard deviation (SD). Statistical significance was assessed by Student's *t* test using Excel 2013 (Microsoft Co., Redmond, USA). Differences with *P* values < 0.01 were considered statistically significant. The protocol of this study was authorized by the Animal Experiment Committee of the National Institute of Health Sciences. The animals were maintained at the animal experimental facility of the National Institute of Health Sciences during the study.

Electrophoresis and western blotting

The suspension of myxospores in PBS ($10^7/\text{ml}$) was mixed with an equal volume of reducing SDS-PAGE sample buffer (Bio-Rad, Hercules, USA). The samples were boiled for 5 min, and 20 μl of each sample (the amount of protein equivalent to 10^5 spores) was loaded per lane on the gel (Mini Protean TGX GEL 4–15%; Bio-Rad). Electrophoretically separated proteins with prestained protein molecular weight markers (Nacalai Tesque, Inc., Kyoto, Japan) were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, USA). After blocking with western blot blocking buffer (Fish gelatin type; Takara Bio, Inc., Japan), the membrane was probed with mouse serum (that was diluted 500-fold with western blot blocking buffer) for 30 min at room temperature and washed with TBST (10 mM Tris-HCl, 100 mM NaCl, 0.2% of Tween 20 [pH 7.5]) three times. The membrane was probed with an alkaline phosphatase-labeled rat monoclonal anti-mouse IgE epsilon chain antibody (Abcam, PLC, UK)—that was diluted 500-fold with western blot blocking buffer—for 30 min at room temperature and then washed with TBST three times. The signals were visualized by means of the BCIP-NBT Staining Kit (Nacalai Tesque, Inc.).

Results and discussion

Food allergy is caused by ingestion of an allergen. Its symptoms include diarrhea and vomiting with a short latent period (within 2 h) (Burks et al. 2012). These symptoms resemble those caused by ingestion of *K. septe mpunctata*. To clarify the involvement of allergy in the foodborne illness caused by *K. septe mpunctata*, we first studied the ability of the myxospore extract to induce IgE production in mice. Given that *K. septe mpunctata* myxospores are ingested by humans orally and cause diarrhea, it is reasonable that mice are administered with myxospores orally. Nonetheless, it has been reported that even egg albumin, a major allergen, does not induce IgE production effectively in mice after oral administration (Dearman et al. 2002; Diesner et al. 2008). In contrast, intraperitoneal exposure to egg albumin induces strong IgE production in mice (Dearman et al. 2002; Diesner et al. 2008; Chen et al. 2013). Therefore, typically, intraperitoneal administration is attempted first for testing of the allergenic potential of an unknown substance (Dearman et al. 2001; Aldemir et al. 2009; Ladics et al. 2010). Furthermore, subsequent subcutaneous injection and the use of an adjuvant are commonly applied to increase sensitivity (Aldemir et al. 2009; Ladics et al. 2010). Because the allergenic potential of our myxospore extract was unknown, we administered samples to mice via intraperitoneal injection, followed by two subcutaneous injections in this study. We injected mice with PBS or egg albumin as a negative and positive control, respectively. The total IgE concentration in serum samples from the negative control group did not increase even after the third immunization (Fig. 1). The total IgE levels in serum samples from the positive control group increased after the second

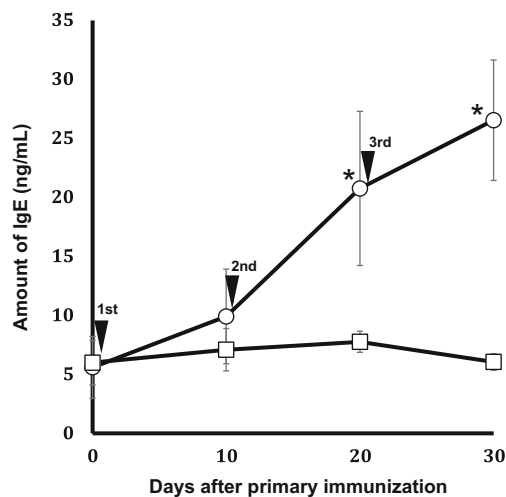


Fig. 1 Time-course of IgE production in BALB/c mice after immunization with the extract of *K. septe mpunctata*. The concentration of IgE was measured by an ELISA at 10, 20, and 30 days after the first immunization with the extract of *K. septe mpunctata* (○; $n = 15$) or PBS (□; $n = 3$). The values are mean $\pm$ SD; * $P < 0.01$ as compared to PBS (Student's t test)

immunization and reached 21.6 ng/ml after the third immunization. When the mice were injected with the myxospore extract, the total IgE concentration in the serum increased significantly ($P < 0.01$) after the second immunization as compared to the negative control (Fig. 1). After the third immunization, the total IgE concentration in the immunized mice reached 26.5 ng/ml and was almost equivalent to that of the positive control, where a major allergic antigen was injected.

We analyzed the specificity of serum IgE by western blotting (Fig. 2). Although the serum samples that were collected from both negative and positive control groups did not react with *K. septe mpunctata* proteins (Fig. 2), IgE antibodies in the serum samples that were collected from myxospore extract-injected mice bound to at least two *Kudoa* proteins with molecular weight between 28 and 36 kDa. This result confirmed that antigen-specific IgE antibodies in mice that were injected with the myxospore extract were indeed raised against *K. septe mpunctata* antigens (Fig. 2). Although it has been reported that a surface substance of a spore, polar capsule, polar filament, or sporoplasm can serve as an antigen (Chase et al. 2001; Ohnishi et al. 2013), the two antigens that were recognized by IgE in this study remain unknown. Further research will be needed to identify the molecules of *K. septe mpunctata* that induce the production of IgE in mice.

A previous report from our laboratory indicated that the invasion of intestinal epithelial cells by sporoplasm is one of the contributing factors of diarrhea (Ohnishi et al. 2013). In that study, the invasion by sporoplasm drastically decreased

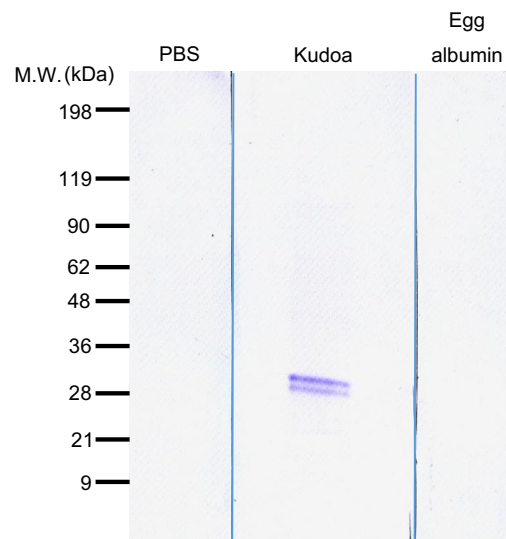


Fig. 2 Specificity of serum samples from *K. septe mpunctata*-injected mice. Electrophoretically separated *K. septe mpunctata* proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was probed with each serum sample from mice that were immunized with the myxospore extract of *K. septe mpunctata*, egg albumin, or PBS. The membrane was probed with an alkaline phosphatase-labeled rat monoclonal anti-mouse IgE epsilon chain antibody. The signals were visualized by means of the BCIP-NBT Staining Kit

the transepithelial electronic resistance of a Caco-2 cell monolayer only 1 h after the inoculation (Ohnishi et al. 2013). Although that infection model well reproduces the clinical signs that are characterized by a short lag phase, it does not include the effects of an immune response on the host (Ohnishi et al. 2013). Other studies such as the mouse models of a *K. septe mpunctata* foodborne illness do not involve quantification of serum IgE either (Kawai et al. 2012). Therefore, the contribution of allergy to the *K. septe mpunctata* foodborne illness has been unclear. Nevertheless, the results of this study show that injection of a myxospore extract induced strong IgE production equivalent to an egg albumin response. Because IgE production is an indicator of an allergic reaction, our findings suggest that a *K. septe mpunctata* antigen is the allergen and that the foodborne illness caused by *K. septe mpunctata* is associated with allergy. The level of *K. septe mpunctata*-specific IgE in patients should be studied to clarify the involvement of allergy in the *K. septe mpunctata* foodborne illness.

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