

# A Study of Allergic Sensitization to *Anisakis* Species in Experimental Mice

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## Abstract

**Title:** A Study of Allergic Sensitization to *Anisakis* Species in Experimental Mice.

**Background:** Hypersensitivity to *Anisakis* species is a worldwide medical problem. The aim of this study was to detect the immunological response in experimental mice, through measurement of Immunoglobulin E (IgE) antibodies in their lymphocytes by flow cytometry, following the ingestion of *Anisakis* crude antigen.

**Methods and Findings:** Sixty Swiss albino mice were divided equally into control and experimental groups, and each of them were further subdivided into five subgroups, six mice each. The percentage of IgE antibodies was measured in the lymphocytes of their splenic suspensions at zero, 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> weeks post inoculation using Fluorescein isothiocyanate (FITC) anti – mouse IgE, and were analyzed by FACS Calibur flow cytometer Becton Dickinson equipped with an argon-ion laser apparatus operating at 488 nm. The percentage of IgE antibodies was enhanced in lymphocytes of animals exposed to *Anisakis* antigen from the first week, peaking three weeks following initial exposure, starting a decline by week five and decreased more by the seventh week, as compared to the control group.

**Conclusions:** the results obtained from this study proved the efficacy of flow cytometry in detecting the sensitization against *Anisakis* species through the measurement of IgE antibodies.

**Keywords:** Allergy, *Anisakis* species, IgE, flow cytometry

## Introduction

Fish play an important role in human nutrition; they are valuable sources of proteins and contain large amounts of healthy fats (so called polyunsaturated fatty acids) and fat-soluble vitamins. However, they are also one of the most common causes of food allergy [1]. Common fish such as herring, tilapia, tuna, salmon and mackerel have all been linked with allergic reactions [1, 2].

Fish allergy is a so-called immunoglobulin E (IgE)-mediated food allergy. It is caused by a reaction to a muscle protein called parvalbumin, present in fish meat. People who are allergic to one type of fish, will also react to other types of fish, because the protein allergens of different fish are similar. This major allergen is extremely stable to heat, which means that boiling or frying of fish does not destroy the allergen [1, 2]. An adverse reaction linked to fish consumption can be also

an IgE-mediated allergy to parasites infecting fish and not to fish meat. Since the parasitic infection of fish is common, this obviously has a diagnostic importance in reactions to seafood. However, a number of parasites, especially helminths, cause synthesis of IgE antibodies against a wide range of proteins released by the parasites during the stage of their life cycle spent in humans. Such parasitic proteins show many immunological similarities to common inhaled or ingested allergens [1, 2]. This parasite-specific IgE antibody is part of the defense system against foreign invaders, and hence protective. At the same time, it also participates in the pathological mechanisms behind a range of inflammatory reactions related to parasite infection [2, 3].

IgE is typically the least abundant isotype. Blood serum IgE level in a normal (non-atopic) individual is only 0.05% of the IgG concentration, it is less than 150 ng/ml, compared to 10 mg/ml for the IgG [4].

*Anisakis* is a nematode parasitizing marine fish, which induces two major problems in humans; infection (anisakiasis), characterized by epigastric pain, nausea, vomiting and diarrhea; and allergic hypersensitivity reactions like urticaria and anaphylaxis. The reactions are mediated by IgE antibodies to *Anisakis* antigens, but were in the past often mistaken as fish allergy [5]. Various studies have shown that 80% of fish could be infested with this parasite [5, 6]. Humans acquire infection with the third stage larva of these parasites by eating raw or undercooked fish. The family Anisakidae includes different genera. There are in fact two types of *Anisakis*, Type I and Type II, and two types of *Pseudoterranova*, often referred to as *cod worm*, and *Contracaecum*. The commonest species is *Anisakis* Type I, namely *Anisakis simplex*. All these species were reported to infect humans in a similar mode as *Anisakis simplex* [7 – 10].

Although cooking at 70 °C or freezing to - 20 °C for 72 hours are believed to destroy the infectivity of this parasite, the allergenic capacity of its denaturated protein is not eliminated [11, 12]. The secretory antigens produced by the worms can induce antibody production against the worms even following ingestion of fully cooked fish containing *Anisakis* antigen [13]. Allergic conjunctivitis and asthma in persons handling fish meals also seem to occur due to the presence of anti-*Anisakis* antibodies [14]. It has been postulated that *Anisakis* allergy may be more prevalent than any specific food allergy in the adult population and comprises as much as 10% of idiopathic anaphylaxis [15].

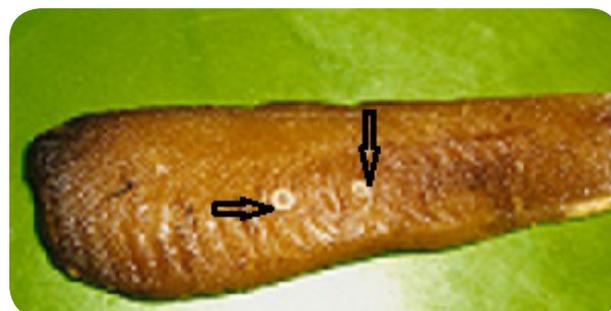
Various immunological methods have been used for the detection of IgE, such as ELISA, immunoblotting, latex-agglutination test and flow cytometry [6, 11, 13, 15, 16]. The latter is a measurement of characteristics of single cells suspended in a flowing saline stream. A focused beam of laser light illuminates each moving cell and light is scattered in all directions, this is picked up by detectors and converted into a suitable form for computer analysis and interpretation. These profiles of cells are normally displayed as dot plots or histograms [17]. To the best of our knowledge, no reports were available about the efficacy of flow cytometry for the detection of the diversity in IgE response against *Anisakis* species parasitizing fish in lymphocytes of splenic suspensions.

The present work was designed to study the immunological response in experimental mice, through measurement of IgE antibodies in their lymphocytes, following the ingestion of *Anisakis* antigen using flow cytometry.

## Methods

### Parasites

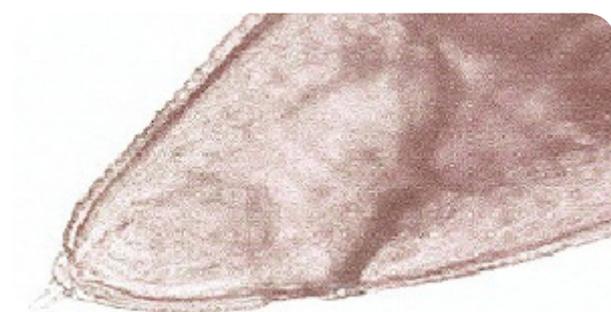
Third stage larvae (L3) of *Anisakis* species were extracted manually from viscera and body cavity of herring purchased in local markets, and were counted individually [6, 9]. They were identi-



**FIGURE 1.** Part of herring fish containing third stage *Anisakis* larvae (L3). The arrows point to the larvae (captured by digital camera).



**FIGURE 2.** Third stage *Anisakis* larva (L3) extracted from herring viscera (captured by digital camera).



**FIGURE 3.** Microscopic picture of third stage *Anisakis* larva showing a spicule at the anterior end (x 100).

fied by being 3-4 cm in length and whitish in colour (**Figure 1, 2**). Under the microscope, each larva possessed a spicule at the anterior end (**Figure 3**).

### Preparation of *Anisakis* antigen

Three hundred larvae were grounded in a mortar with 10 ml phosphate buffer saline (PBS). The mixture was centrifuged for

15 minutes at 4500 x g and the pellet was discarded. The supernatant was separated, and its protein content was 2mg/ml as measured by the method of Bradford [18]. The supernatant was stored at - 20 ° C without any addition as a crude extract [11, 15].

## Animals

The present work was carried out on sixty male Swiss albino mice, three to five weeks of age, weighing 20 to 25 grams. They were kept in suitable cages with perforated covers, fed on standard pellet food and water in addition to libitum. They were free of parasites as shown by repeated stool examination by direct wet saline smear, iodine and Sheather's sugar flotation method. Stool examination was performed before antigen inoculation, as well as weekly post inoculation till the end of the study. Animals were bred in the laboratory of the Parasitology Department. This animal study was approved by the Ethics Committee of Alexandria University. They were divided equally into two main groups:

**Group I:** Control group, included 30 non infected mice.

**Group II:** Experimental group, included 30 mice which were inoculated orally with *Anisakis* antigen by intra-gastric gavage [15, 19]. The dose was calculated to be 0.1 ml / mouse according to a pilot study. In the pilot study, three doses were tested initially; 0.05 ml, 0.1ml and 0.15ml. It was discovered that 0.5 ml was not enough to produce sensitization for the mice, thus it was discarded. On the other hand, both 0.1ml and 0.15 ml were sensitizing to the mice. Thus, we selected the minimal one which is the 0.1ml as an optimal concentration.

Both groups were subdivided into five subgroups (six mice each) according to the duration of sacrifice; zero, 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> weeks post inoculation. All animals were sacrificed by an over dose of ether.

The spleens of mice of all subgroups were aseptically collected at the same duration of sacrifice. They were preserved at - 20° C to be processed for the detection of IgE antibodies in their lymphocytes by flow cytometry [16, 20].

## Preparation of splenic suspensions

Suspensions were prepared by forcing the splenic tissues through a fine wire mesh sieve, using a piston of five ml disposable syringe. This was done to retain the connective tissue capsule of the spleen in the mesh sieve. This was followed by several extensive washes with PBS till a suspension was obtained. All obtained cell suspensions were maintained on ice to preserve their viability.

Lymphocytes were then separated from splenic suspensions by differential centrifugation on a density gradient as follows. In sterile 15 ml conical centrifuge tubes, three ml splenic suspensions were carefully layered over three ml sterile Ficol-

Hypaque, and centrifuged at 400 x g for 30 minutes. The lymphocytes mononuclear cell layer was cautiously collected by a sterile Pasteur pipette and washed thrice in PBS. Each wash was done by centrifugation at 400 x g for ten minutes. The supernatant fluid was aspirated and discarded, after each wash. The cell pellets of each subgroup of mice (6 mice) were collected and pooled together, and were resuspended in a dose of  $5 \times 10^5$  cells / tube in three ml PBS and subjected to immunofluorescent staining. Fluorochrome intensity was measured by monoparameter flow cytometry [16, 20 – 24].

Detection of IgE antibodies was performed using Fluorescein isothiocyanate (FITC) anti – mouse IgE (23G3) PE (eBioscience, San Diego, California). The 23G3 monoclonal antibody reacts only with the heavy chain of mouse IgE, and not with any other class of mouse immunoglobulins.

## Flow cytometry

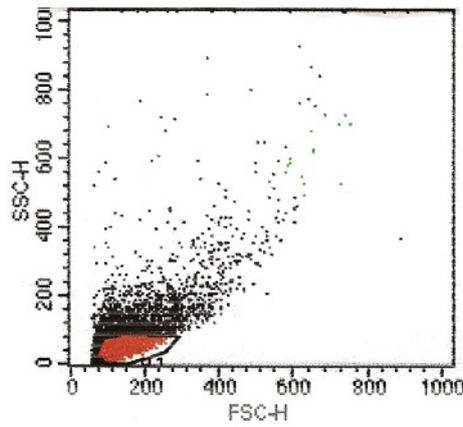
Lymphocyte samples were analyzed on a FACS Calibur flow cytometer Becton Dickinson equipped with an argon-ion laser apparatus operating at 488 nm. The flow cytometer was calibrated by using CalIBRITE beads (Becton Dickinson, Mississauga, Ontario, Canada) and the samples were analyzed using monoparametric (monobasic) histograms. Surface marker analysis was conducted, after gating for forward and side scatter, using Cell Quest software program. It took about two minutes for the apparatus to process each sample and show the results on the computer screen [16, 20, 25].

## Results

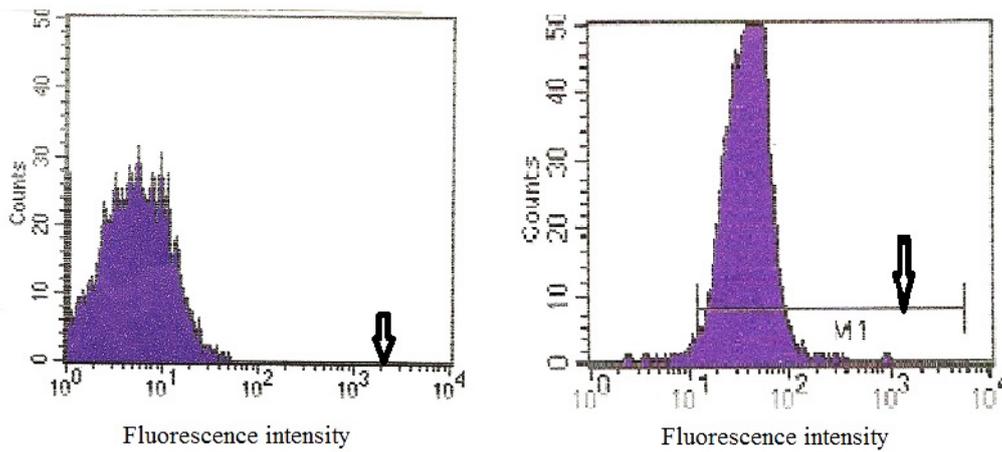
After staining the samples with specific fluorochromes (FITC), they were analyzed using monoparameter flow cytometry. All particles found in the samples appeared on a graph as dot plots (**Figure 4**). Beside this graph, a monobasic histogram appears on the screen of the computer. The x-axis of the histogram represents the intensity of the fluorescence detected by the positive cells according to the Cell Quest program, and the y-axis represents the number of cells read by the apparatus. Examples of negative and positive histograms are shown in figure 5 (a & b) respectively.

The arrow points to the line of the marker. In the control group (negative), the line of the marker was laying on the x-axis of the histogram (**figure 5a**). While in the experimental group (positive), the line of the marker was evident, appearing above the x-axis and passing through the graph (**figure 5b**). The more the line of the marker passes through the graph, the higher the percentage of IgE antibodies.

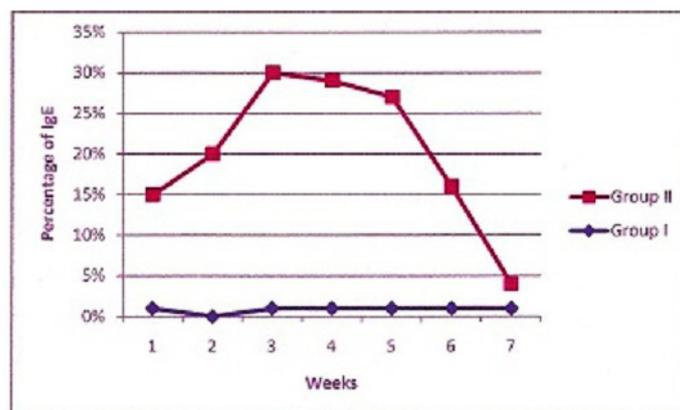
The percentage of IgE antibodies was enhanced in lymphocytes of animals exposed to *Anisakis* antigen from the first week (14%), peaking three weeks following initial exposure



**FIGURE 4.** Flow cytometry graph showing dots representing a positive sample.



**FIGURE 5a y b.** Flow cytometry histogram showing a negative control sample and a positive sample. The arrow points to the line of the marker.



**FIGURE 6.** Time course flow cytometric analysis of IgE in the mice lymphocytes following exposure to *Anisakis* antigen

(29%), and starting to decrease by week five (26%). By the seventh week post-exposure, they declined to (3%). While in the control group, IgE remained more or less at the same level throughout the duration of the study, namely about 1% (Figure 6).

## Discussion

Infection of humans with *Anisakis* species was first described in the 1960s in association with the consumption of raw or undercooked fish. During the 1990s it was realized that the ingestion of dead worms in fish can cause severe hypersensitivity reactions. These may be more prevalent than the infection itself, and this outcome could be associated with food preparations which were previously considered safe. Allergic symptoms are due to the presence of IgE antibodies that constitute an important component of the immune response to the parasite. The in vivo (skin-prick tests) and/or in vitro level of specific IgE are usually measured for diagnosis of sensitization to the parasite, and it is accepted that these determinations are highly sensitive methods [13, 14, 26, 27].

Measurement of IgE antibodies can aid in the diagnosis of parasitosis, as well as, potentially even more important, it can contribute essential information to the investigation of several allergic conditions, including fish allergy [15, 28]. Epidemiological and immunological studies indicate contribution of helminthes infection to the development of asthmatic conditions, depending on a direct relation which was present between the degree of helminthes infection and an increase in peak expiratory flow by the bronchodilator [29]. On the other hand, some authors suggest that the production of the non-specific IgE by helminthes infections can suppress the allergic response to environmental and parasite allergens via protection from mast cell or basophil degranulation by saturating IgE binding sites [30, 31]. However, in our study the *Anisakis* antigen was prepared from dead *Anisakis* larvae. Thus, there is no possibility that the produced IgE was related to parasitic infections. Furthermore, the detected IgE could not be due to a fish allergy caused by a reaction to the parvalbumin, present in fish meat, as the *Anisakis* larvae used in the present study were extracted manually from viscera and body cavity of herring, and no fish meat was given to mice.

In the present work, a murine model of allergy was generated by sensitization with proteins of *Anisakis* larval antigen. This model exhibited characteristics of sensitivity reaction very similar to those observed in humans in the form of enhancement in the IgE level. These findings were also observed by Iglesias *et al.*; 1995 [6] and Baeza *et al.*; 2005 [15], who reported that mouse specific IgE pattern had similarities with that detected in humans. They also reported that the histological and haematological alterations observed in infected mice, are similar to those occurring in humans.

The IgE level was enhanced in lymphocytes of splenic suspensions of animals exposed to *Anisakis* antigen from the first week of exposure, peaking after three weeks and decreased to a low level which was nearly similar to that of the control group at the seventh week post-exposure. This coincided with Akao and Yoshimura, 1989 [13] who reported that using ELISA assay, IgE antibodies were detectable as early as one week, following *Anisakis simplex* infections, and they disappeared as late as 35 days post-infection. They also reported that the antigens involved in IgE production were not affected by freezing or cooking temperatures, enhancing the same IgE pattern as that produced following infection. Furthermore, Manetz and Meade, 1999 [16] recorded an increase in the level of IgE by flow cytometry in the draining lymph nodes and spleen of animals eight days following sensitization with an IgE inducing allergens other than *Anisakis* antigen, and then, the IgE level returned to its normal level within the second month of sensitization.

Several methods have been used for detection of hypersensitivity reactions to *Anisakis simplex* proteins, such as ELISA, immunoblotting, latex-agglutination test [6, 11, 15, 31, 32]. However, few studies applied flow cytometry to diagnose *Anisakis* allergy, but using samples other than lymphocytes. González-Muñoz *et al.*; 2005 [33] detected allergen-induced basophil activation (BAT) by flow cytometry, using whole-blood samples and live basophils, detecting activation-associated membrane markers (CD63) of antigen binding to the IgE high-affinity receptor in response to *Anisakis simplex* crude extract. A significant activation of basophils (98%) was found for patients allergic to *Anisakis simplex* versus controls (3 %).

In the present study, the flow cytometric analysis was very rapid and proved to be a very suitable tool for detecting the lymphocytic IgE antibodies against *Anisakis* allergy. Similar findings about the reliability of the flow cytometry technique have been published by others. Toma *et al.*; 1996 [34] demonstrated that flow cytometric measurement of basophil-bound IgE provided a useful method of analyzing the atopic cell population within the peripheral circulation, and it served as a critical parameter to evaluate the allergic inflammation in vivo. Moreover, Saite-Laudy *et al.*; 2000 [35] reported that flow cytometry showed a high sensitivity and a high specificity in diagnosis of venom allergy.

In conclusion, we have generated a murine model of sensitization to *Anisakis* proteins, which opens new possibilities to study the human allergic reactions to parasite antigens. The results of the present study would explain the high percentage of sensitization against *Anisakis* species, and demonstrate the usefulness of flow cytometry for immunological diagnosis of *Anisakis* allergy.

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