

Anti-*Anisakis* sp. antibodies in serum of healthy subjects. Relationship with $\alpha\beta$ and $\gamma\delta$ T cells

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Abstract

Anisakiosis is nowadays one of the nematodoses more prevalent in Spain, with rates that oscillate between 0.43% in Galicia (N.W. Spain), and 15.7% and 22.1% in inland and southern regions, respectively. Likewise, it has been proved that *Anisakis* larvae have developed mechanisms to modulate the dichotomy of the host immune response for their own benefit. The experimental hypothesis of the present study was that *Anisakis* sp. larval products can be mediators of immune suppression and induce changes on the populations of $\alpha\beta$ + and $\gamma\delta$ + T cells. In the present study we determined the levels of anti-*Anisakis* antibodies in the serum of healthy people, and their relationship with the B and T cell subsets. Levels of anti-*Anisakis* antibodies (Ig's, IgG, IgM, IgA and IgE) were measured by ELISA, while B and T cell subsets were studied by flow cytometry. Cells were labelled with monoclonal antibodies against CD45, CD4, CD8, CD56, CD3, CD19, TCR $\alpha\beta$ and TCR $\gamma\delta$. All the specific isotypes studied were negatively correlated with NKT cell rates with the exception of IgG. A previous contact with *Anisakis* was related to a decrease in CD56+ $\alpha\beta$ + and all $\gamma\delta$ + T cell subsets. The CD3+ $\gamma\delta$ + population was lower in the group of subjects that showed IgA anti-*Anisakis*. We observed an inverse correlation among $\alpha\beta$ - $\gamma\delta$ NKT cells and anti-*Anisakis* sp. antibodies. CD3+CD56+ cells showed a significant decrease in the group of anti-*Anisakis* positive subjects. This fact was especially significant with CD3+CD56+ $\gamma\delta$ + cells in the case of the anti-*Anisakis* IgA positive group.

Keywords

Anisakis, T cells, NKT cells, CD56+ $\alpha\beta$ + cells, CD3+CD8+ $\gamma\delta$ + cells, CD3+CD56+ $\gamma\delta$ + cells

Introduction

Anisakiosis (infection caused by anisakids, especially *Anisakis* sp.) is nowadays one of the nematodoses (infections caused by parasitic nematodes) more prevalent in Spain. Previous studies have shown that anisakiosis seroprevalence is very variable among different Spanish regions, with rates that oscillate between 0.43% in Galicia (N.W. Spain), and 15.7% and 22.1% in inland and southern regions, respectively (Valiñas *et al.* 2001; Fernández de Corres *et al.* 2001; del Rey Moreno *et al.* 2006). Using the monoclonal antibody UA3 specific of *Anisakis simplex*, the seroprevalence of specific IgE anti-*A. simplex* was evaluated in Madrid as a representation of Spanish population since in such city is approximately inhabiting 10% of the same (Puente *et al.* 2008). In spite of, all the in-

terviewed subjects related a frequent consumption of raw fish, the prevalence of positive cases was 12.4% (11.7% of healthy individuals and 16% of patients with non digestive/non allergy pathologies). The positivity was more prevalent among the subjects that frequently consumed fresh and, perhaps, little cooked fish than among consumers of frozen, boiled or baked fish. These results, in addition to the elevated rates of fish consumption, support the interest of researching in this area of Parasitology.

The *Anisakis* sp. third stage larvae invade the gastrointestinal mucosa by the mechanical rupture of the tissues and the secretion of potent proteolytic enzymes able to degrade the extracellular matrix (Morris and Sakanari, 1994). *Anisakis* larvae have developed mechanisms to modulate the dichotomy of the host immune response for their own benefit. Their an-

ticoagulant action plays an important role during the invasion of the gastric or intestinal mucosa with a biological significance in the infected patients (Perteguer *et al.* 1996; Valdivielso *et al.* 2015). Their suppression of Th1 responses was proved by their capacity to inhibit nitric oxide production by macrophages (Cuéllar *et al.* 1998), being also confirmed their anti-inflammatory potential by the presence of IL-4-like molecules (Cuéllar *et al.* 2001) and a macrophage migration inhibitory factor homologous (Park *et al.* 2009). Likewise, *A. simplex* larval antigens have immunomodulatory activity on the complement system (García-Hernández *et al.* 2007; 2009).

Using anisakiosis murine experimental models, we have previously observed different responses with a susceptible behaviour in BALB/c mice and resistant in the C57BL strain (Perteguer and Cuéllar, 1998). Regarding BALB/c mice, more prominent gastric and/or intestinal lesions were observed in animals which were orally infected with *Anisakis* living larvae. These inflammatory processes were inhibited by the previous injection of larval antigen that also provoked a significant reduction of CD45+ and CD8+ cells. Likewise, total larval antigen was able to induce an inhibition of the *in vitro* proliferation of T cells (Perteguer *et al.* 2001).

In anisakiosis cases, the larva survives for two or three weeks after penetrating into submucosa of the digestive tract of its host. From 6 to 10 days after larval penetration a granuloma appears around the larva, which later, still alive, sensitizes the newborn granuloma with their metabolic products. After the larva dies, its cuticle starts to disintegrate and the cells and the exudates released by the parasite directly react with the sensitized granuloma producing an inflammation around the death larva (Jones *et al.* 1990). The antigens liberated by the degenerated larva may be captured and carried by dendritic cells through lymphatic vessels which could have previously been sensitized by the material released by the living larva, forming the core of a typical secondary response after the oral ingestion of helminth parasites, which typically includes the activation of neutrophils, macrophages of the tissues, monocytes, dendritic cells, basophils and eosinophils. The prototypical response to helminths includes the production of the cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 and the induction of antigen-specific IgG1, IgG4 and IgE related to Th2 lymphocyte populations (Nutman, 2015).

From the discovery of a new type of T cell receptor (TCR- $\gamma\delta$ receptor), T cells are classified in two major groups ($\alpha\beta$ + and $\gamma\delta$ + T cells). Since then, $\gamma\delta$ + T cells are cause of incessant studies that show their surprising immune functions (Payne, 2016). Although in the peripheral blood of adult donors there are approximately 90%–95% $\alpha\beta$ + T cells and 5%–10% $\gamma\delta$ + T cells among CD3+ T cells, more than 50% of intraepithelial lymphocytes are $\gamma\delta$ + T cells (Goodman and Lefrançois, 1988). Therefore, this receptor is credited with a major role on the innate immune response as a first line of defence.

Clearly most pathogens enter in our body through mucous membranes, being the gut one of the most important systems (Thaiss *et al.* 2016). Thus, it is not a surprise that gut-associ-

ated lymphoid tissues contain the largest pool (80%–85%) of immunocompetent cells in the human body where $\gamma\delta$ + T cells are particularly important (Brandtzaeg *et al.* 1989).

The experimental hypothesis of the present study was that *Anisakis* sp. larval products can be mediators of immune suppression as they can interact directly with the dendritic cells of the intestinal mucosa which will produce tolerogenic signals able to induce changes on the populations of $\alpha\beta$ + and $\gamma\delta$ + T cells. Our goal was to analyze the levels of anti-*Anisakis* antibodies in the serum of healthy persons, and its relation with the B cells and T cell subsets.

Materials and Methods

Study population

One hundred and thirty-two healthy subjects were selected among relatives of patients admitted in the Arnau de Vilanova Hospital, in Valencia (Spain), as well as the family of workers from the same hospital. 69 were male (52.3%) and 63 female (47.7%) with a mean age of 63.1 ± 20.6 and 59.1 ± 18.1 in the case of male and female, respectively. They had to meet the following criteria: absence of acute infections, inflammatory, autoimmune or immunodeficiency diseases, and have not received immunosuppressive or antibiotic treatment or any kind of vaccine during the last six months.

Patients were recruited from January to December of 2013. Each participant in the study signed an informed consent form, and our Hospital's Ethics Committee for Clinical Research approved the study (document n° 20/2012).

Variables studied

The following variables were recorded: age and gender, lymphocyte subsets, including CD3+, CD4+, CD8+, CD56+, CD19+, CD3+ $\alpha\beta$ +, CD4+ $\alpha\beta$ +, CD8+ $\alpha\beta$ +, CD56+ $\alpha\beta$ +, CD3+ $\gamma\delta$ +, CD8+ $\gamma\delta$ +, CD4-CD8- $\gamma\delta$ + and CD56+ $\gamma\delta$ + cells; and anti-*Anisakis* antibodies.

Analytical determination of B cells and T cell subsets

Peripheral blood samples obtained by venipuncture were collected in K3-EDTA anticoagulant and processed within six hours of collection. Blood cell counts were performed using the Coulter LH750 automated hematology analyzer (Beckman Coulter, Fullerton, CA).

Whole blood was stained using direct immunofluorescence and simultaneous quadruple labelling with the following monoclonal antibodies: CD45, CD4, CD8, CD56, CD3, CD19, TCR $\alpha\beta$ and TCR $\gamma\delta$. Monoclonal antibodies were conjugated with fluorescein iso-thiocyanate (FITC), phycoerythrin (PE), phycoerythrin-Texas red (ECD), and R-phycoerythrin-cyanine 5 (PC5).

The $\gamma\delta$ T lymphocyte populations were analyzed with PC5

conjugated anti-human $\gamma\delta$ TCR (Beckman Coulter, Miami, USA [clone: IMMU510]).

The $\alpha\beta$ T lymphocyte populations were analyzed with PC5 conjugated anti-human $\alpha\beta$ TCR (Beckman Coulter [clone: IP26A]).

For each sample prepared four polyethylene tubes were labeled. Monoclonal antibodies were added to each tube, 20 μ l for FITC and PE conjugated antibodies and 10 μ l for ECD and PC5 conjugated antibodies. Following this, 100 μ l of blood sample were added and gentle vortex agitation was performed. This was then incubated at room temperature in a dark place for 20 minutes. Red cell lysis was performed then with a simultaneous fix and lyse reagent, VersaLyse (BeckmanCoulter, Inc). The fix and lyse solution was added to the tubes, then gentle vortex agitation was performed and 15 minutes incubation at room temperature in a dark place followed. After this, 5 minutes centrifugation at 150 g was performed. Then supernatant was removed and 3 ml of PBS were added, this washing step was repeated three times. Finally 0.5 ml of PBS were added before acquisition.

Fluorescence analysis was performed using a Beckman-Coulter multiparameter flow cytometry analyzer, Cytomics FC 500, Florida (USA) and later analyzed with CXP Software. A minimum of 30.000 events were measured.

Anti-*Anisakis* sp. antibodies

Third-stage larvae (L3) of *Anisakis* were extracted from blue whiting (*Micromesistius poutassou*). For the preparation of the crude extract, L3 were homogenised following sonication and extraction in PBS as previously described (García-Palacios *et al.* 1996).

Each well of 96-well microtitre plates (Costar, Corning, NY, USA) was sensitized by the addition of 10 μ g/ml of larval antigen. Duplicate dilutions of human sera at 1/100 in PBS-Tween, containing 0.1% BSA were added and incubated. Horse radish peroxidase (HRP) conjugate goat anti-human Ig's (1/10000), IgM (1/8000), IgG (1/8000) or IgA (1/3000) (Biosource International, Camarillo, CA, USA) were used (Daschner *et al.* 2002; Gutiérrez and Cuéllar, 2002).

For the IgE determination test sera were added in duplicate at a 1/2 dilution. A murine monoclonal antibody against an epsilon human IgE chain (1/1000) (IgG1 κ , E21A11, IN-GENASA, Madrid, Spain) was added and incubated, followed by a goat anti-mouse IgG1 (gamma) HRP conjugate (1/1000) (Life Technologies, Grand Island, NY) (Gutiérrez and Cuéllar, 2002, Martínez de Velasco *et al.* 2006).

Statistical Analysis

The assumption of a normal distribution for continuous variables was verified by using graphic examination and Kolmogorov-Smirnov test with a Lilliefors's significance correction. When normality was assumed, the Student *t* or ANOVA

tests were used to compare the quantitative variable mean values. When the hypothesis of normality for the quantitative variables was not accepted, the nonparametric Mann-Whitney *U* test and Kruskal-Wallis test were used. Correlation studies using Pearson's correlation coefficient were performed to compare immunoglobulin levels and T cell subsets.

Significance was defined as a *p*-value less than 0.05. Data were analyzed using the statistical software SPSS, version 19.

Results

One hundred and thirty-two healthy subjects were analysed. The mean age of the subjects included in this study was 59.6 $\pm$ 19.7 years, minimum 18, maximum 95 years. Sixty nine subjects were males (52.3%).

Anti-*Anisakis* sp. antibody levels

In the Table I the mean values of anti-*Anisakis* antibody levels are present. The levels of specific IgM observed in the group of females were significantly higher than those observed in the male group. No significant differences were observed between sexes for the rest of antibodies tested.

The anti-*Anisakis* antibody levels correlated negatively with the age in the case of total immunoglobulins and IgM (Spearman rho, -0.320 , $p < 0.001$ and Spearman rho, -0.373 , $p < 0.001$, respectively). According to the age groups (Table II), the anti-*Anisakis* total immunoglobulins were higher in subjects younger than 55 years and decrease significantly from that age (56–75 years, $p = 0.008$ and 76–95 years, $p = 0.005$). This decrease was more patent in the case of the anti-*Anisakis* IgM levels, with significant differences in the groups of 56–75 years ($p = 0.021$) and 76–95 years ($p = 0.002$) compared to the younger groups (Kruskal-Wallis test). However, in the case of IgG and IgE levels the opposite effect was observed. The anti-*Anisakis* specific IgE levels increased significantly in the group of 56–75 years ($p = 0.022$) and 76–95 years ($p = 0.033$) respect to the 15–35 years group; and

Table I. Anti-*Anisakis* simplex antibody levels according to gender in healthy subjects

	Male	Female	<i>p</i> value
Ig's	0.651 $\pm$ 0.364	0.692 $\pm$ 0.241	N.S.
IgG	0.844 $\pm$ 0.505	0.808 $\pm$ 0.453	N.S.
IgM	0.806 $\pm$ 0.494	0.904 $\pm$ 0.511	< 0.001
IgA	0.674 $\pm$ 0.471	0.723 $\pm$ 0.520	N.S.
IgE	0.778 $\pm$ 0.954	0.813 $\pm$ 0.903	N.S.

Results are expressed as Absorbance values at 490 nm. To compare the different immunoglobulin isotypes logarithms were calculated after multiplying values by 10 (Ig's, IgG, IgM and IgA; dilution 1/100) or 500 (IgE; dilution 1/2). Ig's: total immunoglobulins. N.S.: not significant. mean $\pm$ standar deviation

Table II. Anti-*Anisakis* antibody levels according to age-groups. 15–35 years (N = 23), 36–55 (N = 28), 56–75 (N = 46), 76–95 (N = 35)

	15–35	36–55	56–75	76–95
Ig's	0.744 ± 0.279	0.728 ± 0.362	0.628 ± 0.230	0.621 ± 0.322
IgG	0.811 ± 0.462	0.816 ± 0.477	0.795 ± 0.398	0.877 ± 0.568
IgM	0.914 ± 0.447	0.923 ± 0.505	0.839 ± 0.556	0.766 ± 0.431
IgA	0.610 ± 0.255	0.766 ± 0.591	0.688 ± 0.505	0.706 ± 0.491
IgE	0.477 ± 0.699	0.602 ± 0.699	0.903 ± 0.699	0.778 ± 1.000

Results are expressed as Absorbance values at 490 nm. To compare the different immunoglobulin isotypes logarithms were calculated after multiplying values by 10 (Ig's, IgG, IgM and IgA; dilution 1/100) or 500 (IgE; dilution 1/2). Ig's: total immunoglobulins. mean ± standar deviation

statistical significance between the groups of 56–75 and 76–95 years ($p = 0.044$). The anti-*Anisakis* specific IgG levels increased in the group of 76–95 years tending to be significant ($p = 0.066$). The IgA anti-*Anisakis* increased slightly in the group of 36–55 years although without a statistical significance (Kruskal-Wallis test).

Table III. Correlations among NKT cells and anti-*Anisakis* antibody levels

	CD3+ CD56+	CD3+ CD56+αβ+	CD3+ CD56+γδ+	CD19+
Ig's	-0.443	-0.292	-0.280	–
<i>p</i> value	<0.001	0.001	0.002	–
IgM	-0.439	-0.247	-0.214	+0.250
<i>p</i> value	<0.001	0.005	0.018	0.004
IgE	-0.234	–	–	–
<i>p</i> value	0.034	–	–	–
IgA	-0.227	-0.301	-0.190	–
<i>p</i> value	0.009	<0.001	0.036	–

Spearman rho test was used. Ig's: total immunoglobulins

Table IV. Frequency of CD19+ cells according to positive and negative anti-*Anisakis simplex* specific antibodies

(N = Positive)	Positive	Negative	Significance (<i>p</i>)
Ig's (20)	251.0 ± 131.4	215.5 ± 144.1	0.198
IgG (17)	194.6 ± 117.9	224.7 ± 145.7	0.519
IgM (15)	260.0 ± 98.7	215.8 ± 146.6	0.051
IgE (9)	221.9 ± 174.8	220.8 ± 140.6	0.738
IgA (22)	232.8 ± 153.3	218.5 ± 140.7	0.700

B cells are expressed: cells/mm³. mean ± standar deviation. Mann-Whitney U test. Ig's: total immunoglobulins

αβ-γδ T cell and B cell correlations with anti-*Anisakis* specific antibody levels

T cell subset values were statistically analyzed with respect to anti-*Anisakis* antibody levels. As it is described in the Table III, all the specific isotypes studied are negatively correlated with NKT cell rates with the exception of anti-*Anisakis* IgG levels. On the other hand, CD19 expression levels correlated positively with serum specific IgM.

Positive antibody levels against *Anisakis* and T cell subsets

In order to carry out the comparison of the quantitative variable mean values subjects were divided in two groups according to their anti-*Anisakis* antibody levels. Values higher than the mean of the Optical Density (OD) of 132 studied sera obtained for each immunoglobulin plus once their respective standard deviation were considered as positive. The percentages of positive for total immunoglobulins, IgG, IgM, IgE and IgA were 15.2%, 12.9%, 11.4%, 6.8% and 16.7%, respectively. The significant frequencies of T cells according to positive or negative anti-*Anisakis* antibodies are shown in the Figure 1. The anti-*Anisakis* positivity was related with a decrease of CD56+αβ+ and all γδ+ T cell subsets especially in the case of anti-*Anisakis* IgA. In the case of CD19+ cells a great frequency was observed in the group of detectable levels of IgM anti-*Anisakis* antibodies which was near to significance ($p = 0.051$) (Table IV). The levels of the rest of antibodies tested did not affect the number of B cells.

When the studied subject were divided in two groups (positive and negative) according to their anti-*Anisakis* antibody levels, the CD56+γδ+/CD3+ ratio (0.0083 ± 0.0106) in the negative group was significantly higher than in the positive groups (0.0036 ± 0.0036) ($p = 0.019$). Interestingly, in the case of anti-*Anisakis* specific IgA, we found an increase in the CD56+γδ+/CD3+ ratio in the negative group compared to the positive group (0.0084 ± 0.0108 and 0.0038 ± 0.0034 , respectively) ($p = 0.001$). In addition, our *Anisakis* IgA negative patients showed a higher CD8+γδ+/CD3+ ratio than the positive (0.0131 ± 0.0135 and 0.0088 ± 0.0072 , respectively) ($p = 0.039$). However, there were no differences

in the case of the rest of specific antibodies tested (data not shown).

Discussion

This is the first study about the relationship among anti-*Anisakis* sp. antibody levels in sera from healthy subjects with $\alpha\beta^+$ and $\gamma\delta^+$ T cells. We observed a significant difference in the case of CD3+ $\gamma\delta^+$ population which was lower in the group of subjects that showed detectable levels of IgA antibodies against larval *Anisakis* antigen. This fact was especially significant in the CD3+CD8+ $\gamma\delta^+$ subset. Interestingly, Borrelli *et al.* (2010) observed an increase of intraepithelial $\gamma\delta^+$ lymphocytes in patients with IgA deficiency suggesting a compensatory mechanism of surface protection due to the lack of this important defence mechanism of the mucosal surface. These authors observed that patients with selective IgA deficiency showed higher $\gamma\delta^+$ /CD3+ ratios. In our study for the case of anti-*Anisakis* specific IgA, we found a significant increase of the CD56+ $\gamma\delta^+$ /CD3+ ratio in the *Anisakis* IgA

negative group compared to the positive subjects. Likewise, the *Anisakis* IgA negative patients showed a higher CD8+ $\gamma\delta^+$ /CD3+ ratio than the positive.

In our positive subjects anti-*Anisakis* IgA can combine with antigens that have been inoculated in the *lamina propria* by the living larvae and subsequently the density of intraepithelial lymphocytes does not need to be increased compared to the negative subjects. It is interesting that Shen *et al.* (2000) demonstrated a close correlation between IL-6 mRNA+ and $\gamma\delta^+$ cells in *Trichostrongylus colubriformis*-immunized sheep but the increase of IL-6 mRNA expression was not correlated with IgA induction.

So far no one has reported that the stimulation of anti-*Anisakis* sp. specific antibodies is related to NKT cells. All the specific immunoglobulin classes measured by ELISA, with the exception of the IgG, were negatively correlated with NKT cells, both $\alpha\beta^+$ and $\gamma\delta^+$ T subsets. In a previous work we observed that in a healthy group of subjects, anti-*Encephalitozoon cuniculi* antibodies were negatively correlated with CD3+CD56+ $\alpha\beta^+$ T cell values, in both IgE and IgG isotypes. Likewise, CD3+CD56+ $\gamma\delta^+$ T cells were neg-

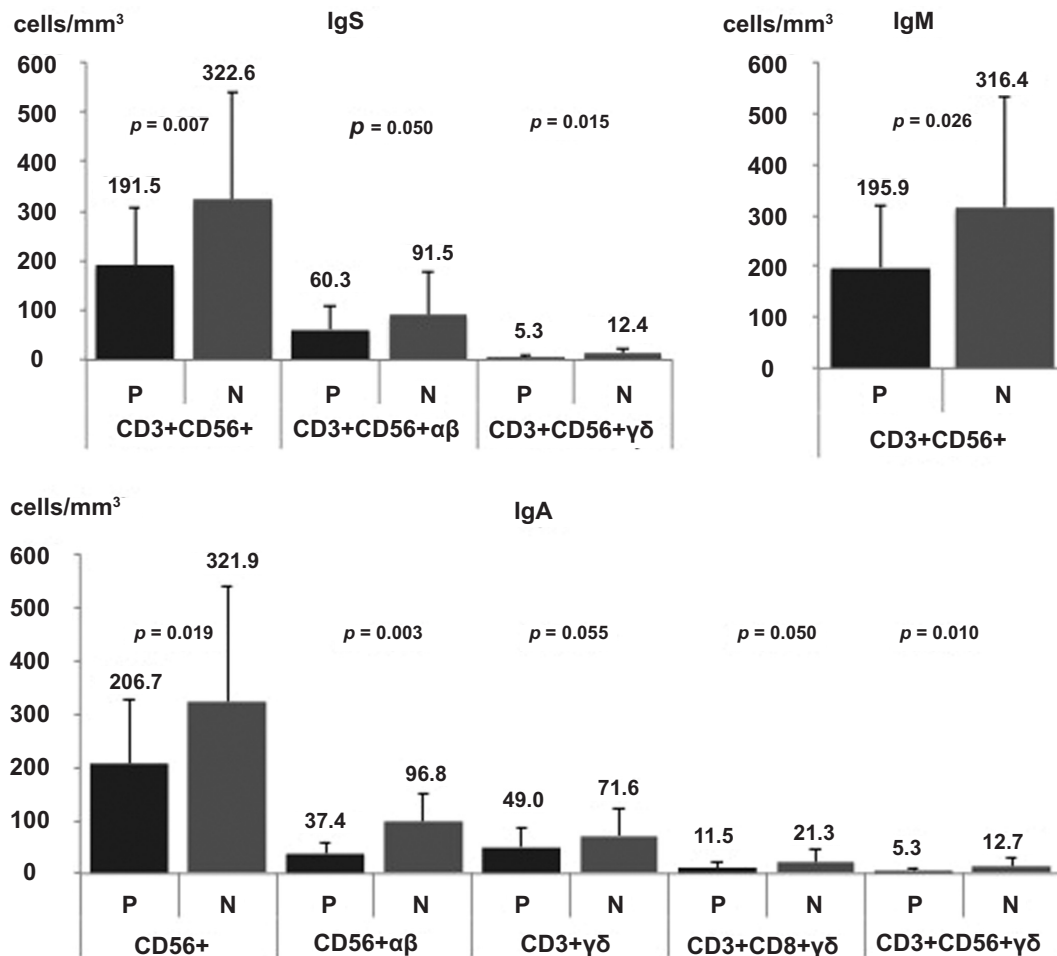


Fig. 1. Significant frequency of T cell subsets according to positive (P) and negative (N) total immunoglobulins (Ig's), IgA and IgM anti-*Anisakis* antibodies. Values of T cell subsets are expressed/mm³. p value, Mann-Whitney U test

atively correlated with IgE anti-*E. cuniculi*, an opportunistic intracellular pathogen (Andreu-Ballester *et al.* 2014). Since NKT cells recognize glycolipid antigens presented by CD1d molecules, but not conventional MHC molecules, it is thought that NKT cells act as an interface between the innate and adaptive systems, to mediate efficient and augmented immune responses, e.g. initiating T cell responses to non-peptide antigens (Fuji *et al.* 2013). Curiously, NKT TCR recognizes the antigen in a similar manner to $\gamma\delta$ T cells and the role of NKT cells expressing the $\gamma\delta$ receptor is unknown. Although the best characterized NKT cell subset expresses CD3 and has a unique $\alpha\beta$ TCR, it is necessary to study in depth the role of $\gamma\delta$ NKT cells in the immune responses against pathogens. Perhaps many of the studies conducted on NKT cells actually include functions of $\gamma\delta$ NKT cells because these authors have not differentiated between $\alpha\beta$ and $\gamma\delta$ T receptors.

In the present study, we observed an inverse correlation among $\alpha\beta$ - $\gamma\delta$ NKT cells and anti-*Anisakis* sp. antibodies. Such correlation could indicate the critical role of these cells of the innate immunity in the protection against this parasite. This is in line with the decrease of NKT cells in patients with anti-*Anisakis* sp. positive antibodies, especially with the decline of $\gamma\delta$ T cells. Both IgA and $\gamma\delta$ T cells are essential parts in the mucosal defence (Mantis *et al.* 2011; Kabelitz *et al.* 2005). CD3+CD56+ cells showed a significant decrease in the group of anti-*Anisakis* positive subjects (total immunoglobulins and IgM). Green *et al.* (2007) observed an inverse correlation between the frequencies of NKT cells and the level of IgG and IgM in relatives of patients with systemic lupus erythematosus. The authors suggested a role for these cells in regulating immunoglobulin levels. CD3+CD56+ $\alpha\beta$ cells also showed a significant decrease in the group of anti-*Anisakis* positive subjects. However, according to Koyama (2002) NK1.1+ cells are not involved in protection against *Trichuris muris* and are not required for the Th2 responses induced by infections with parasites. Nevertheless, iNKT cell percentages are diet-dependent and directly related to atrophy degree (Calleja *et al.* 2011), so it is necessary to take into account this fact in the case of a food-borne parasite as *Anisakis* whose living larvae were trying to penetrate across the intestinal mucosa. Finally, CD3+CD56+ $\gamma\delta$ cells showed a significant decrease in the group of anti-*Anisakis* positive subjects especially in the case of the IgA positive group. Although there is no information regarding this non-conventional population on intestinal parasite-associated diseases, their involvement in the immunopathological mechanisms and defence against helminths need to be studied in depth. Interestingly, Fujimiya *et al.* (1997) observed that IL-12 was able to induce activation of peripheral blood CD3+CD56+ $\gamma\delta$ cells, so that the immunomodulatory effects exert by the products released by the larvae in the group of positive healthy subjects could be producing the opposite effects and consequently causing the diminution of this cell subset.

NKT cells produce great quantities of IL-4 in response to nematode antigens providing the initial source of this cytokine

for Th2 priming (Yoshimoto *et al.* 1995). Since IL-4 is involved in the generation of allergic responses the examination of the involvement of NKT cells in Th2 responses and IL-4 production in response to physiological activation by *Anisakis* infections is an interesting issue. Previous observations on the ability of *Anisakis* L3 to produce extremely fast antibody responses (Perteguer and Cuéllar, 1998; Daschner *et al.* 2002) allow the hypothesis that NKT cells produce IL-4 in the early phase of *Anisakis* infection resulting in the induction of large additional quantities of IL-4 as a consequence of an early induction of Th2 cell differentiation.

In conclusion, NKT cells appear to be related to the immune response against *Anisakis*. Therefore, researching on this area should continue in order to improve our knowledge on it. This would entail a better understanding of the immunological mechanism developed against this parasite, and may lead to new treatments of allergic diseases.

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