

The effect of anti-*Anisakis simplex* antibody levels on C3 and C4 complement components in human sera

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Abstract

Previously, an *in vitro* effect was observed on the complement system not only of the excretory-secretory products but also of somatic antigens from L3 *Anisakis simplex* larvae. In the present work the effect of anti-*A. simplex* specific antibodies on C3 and C4 levels in human sera was investigated. Up to 309 samples of sera were tested to determine levels of C3 and C4 and anti-*A. simplex* antibodies, including immunoglobulins IgG, IgM, IgA and IgE. Significant differences were observed between levels of C3 and C4 and all immunoglobulins except for IgE. In the case of immunoglobulins, the probability that an anti-*A. simplex* positive subject has a C3 deficiency was 3.8 times higher than a subject without specific antibodies. In conclusion, an association between elevated levels of anti-*A. simplex* antibodies and C3 and C4 deficiency was demonstrated.

Introduction

The complement system participates in inflammatory processes through several proteins, mainly C3a and C5a anaphylatoxins (Frank & Fries, 1991). Parasites display a variety of mechanisms for effective evasion of the humoral and cellular immune responses of the host. Parasites have developed a large number of specific strategies to overcome immune defence, among others by avoiding either recognition or eradication by complement. *Schistosoma mansoni* releases the glycocalyx coat, a strong activator of the alternative pathway (AP) of the complement system (Marikovsky *et al.*, 1990). *Naegleria fowleri* uses membrane vesiculation to remove the membrane attack complex (C5b-9) (Toney & Marciano-Cabral, 1994). Paramyosins of helminth parasites, such as *Taenia solium* and *S. mansoni*, induce complement inhibition at the C1 level, being directed at the initiation of the classical pathway (CP) (Laclette *et al.*, 1992). A C9

cross-reactive protein from *Trypanosoma cruzi* forms pores in the membrane, which contributes to the infection process (Andrews *et al.*, 1990). Mannan-binding-lectin (MBL) bound specifically to the components of crude extract and excretory-secretory (ES) products of *Trichinella spiralis* muscle larvae (Gruden-Movsesijan *et al.*, 2003). Carter *et al.* (2007) observed a role for MBL-A in the development of humoral immunity against *Brugia malayi* and its deficiency renders mice more susceptible to the infection. MBL also interacts with different parasites, such as *Leishmania* spp. (Peters *et al.*, 1997), *T. cruzi* (Kahn *et al.*, 1996) and *S. mansoni* (Klabunde *et al.*, 2000). *Schistosoma* complement C2 receptor inhibitor trispanning (CRIT; formerly, trispanning orphan receptor), like C1-inhibitor, inhibits C1-mediated cleavage of C2, so preventing formation of the CP C3 convertase, C4b2a (Inal, 2005). Inal (2005) found a CRIT from *Trypanosoma* closely related to homologous molecules in human, rat and cod. The use of complement may facilitate parasite survival. Thus, sequestration of host factor H in the hydatid cyst wall inhibits C3b deposition by *Echinococcus granulosus* (Díaz *et al.*, 1997).

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Anisakis simplex is a nematode parasite which is acquired by human patients when eating raw or undercooked fish or seafood that contains third-stage larvae (L3), producing a disease known as anisakiosis. *Anisakis* L3 can be invasive, penetrating the host's stomach or the intestinal wall, provoking inflammation (Morris & Sakanari, 1994). Anisakiosis is presently one of the most prevalent nematode diseases in Spain; the high prevalence of the parasite in Madrid is related to consumption of raw or poorly cooked fish (Puente *et al.*, 2008). Our previous studies revealed that larval products of *A. simplex* L3 (crude extracts (CE) and ES products) cause a dose-dependent inhibition of complement haemolytic activity, both for the AP and for the CP. The consumption of complement factors was demonstrated in the assays shortening the preincubation period compared with the standard time of 30 min at 37°C. In this previous work, a possible activation of the complement by incubating appropriate sera dilutions with *A. simplex* CE and ES concentrations for 0 and 15 min was studied (García-Hernández *et al.*, 2007). When the effect of *A. simplex* larval products on the C3 complement component was tested using C3-deficient sera, a significant decrease in the inhibitory percentages produced by *A. simplex* products on the haemolysis mediated by the activation of the complement pathways was observed only in the CP, but not in the AP. AP and CP activities were determined using a modified version of the microassay described previously (Halkes *et al.*, 1997). This fact could indicate that *A. simplex* larval products also exhibit activity on components that are exclusive of CP (García-Hernández *et al.*, 2007). Our findings on the activity of *A. simplex* larval products on the complement system led us to examine a possible interference with various components of the complement cascade (García-Hernández *et al.*, 2007). When the assays were performed using C5-, C6-, C7-, C8- and C9-deficient sera, *A. simplex* larval products had no impact on the complement system, whereas CE and ES products influenced the C2 complement component (García-Hernández *et al.*, 2009). In this case, among early complement components, *A. simplex* larval products only produced a dose-dependent inhibition of lysis when C2-deficient serum was tested. Besides, *A. simplex* ES products showed a stronger inhibition than CE. Thus, the inhibitory percentages achieved with ES products in the C2-deficient serum assay were similar to those obtained with the control test. These results were in agreement with the loss of haemolysis inhibition activity by *A. simplex* larval products using C3-deficient sera on the CP, because the protein C2 is exclusive of this pathway.

The aim of our study was to evaluate C3 and C4 levels in human sera from patients positive for *A. simplex* antibodies compared to a control group of non-*A. simplex*-positive subjects. The anti-*A. simplex* antibody levels were evaluated with respect to the increase or decrease of C3 and C4 complement fractions.

Materials and methods

Serum samples

Up to 309 samples of sera from residents of Madrid ($n = 149$) or Valencia ($n = 160$), Spain (age 20–85 years,

mean 48.4 years) were randomly selected. Prior signed consent was obtained from all participants in the study. This research was approved by the Clinical Ethics Committees of the hospitals (Central de la Defensa, Madrid and Arnau de Vilanova, Valencia, Spain).

Antigens and specific antibody levels

For the preparation of the CE antigen, *A. simplex* L3 were extracted from blue whiting (*Micromesistius poutasou*) and homogenized following sonication and extraction in phosphate-buffered saline (PBS) as described previously (García-Palacios *et al.*, 1996).

Each well of 96-well microtitre plates (Costar, Corning, New York, USA) was sensitized by the addition of *A. simplex* CE antigen. Duplicate dilutions of sera at 1/100 in PBS-Tween, containing 0.1% bovine serum albumin (BSA) were added and incubated. Horseradish peroxidase (HRP)-conjugated goat anti-human immunoglobulins, IgM, IgG or IgA (Biosource International, Camarillo, California, USA) were used (Daschner *et al.*, 2002; Gutiérrez & Cuéllar, 2002). For the IgE determination, test sera at 1/2 dilution were added in duplicate. A murine monoclonal antibody against an epsilon human IgE chain (IgG1 κ , E21A11, INGENASA, Madrid, Spain) was added and incubated, followed by a goat anti-mouse IgG1 (gamma) HRP conjugate (CALTAG Laboratories,

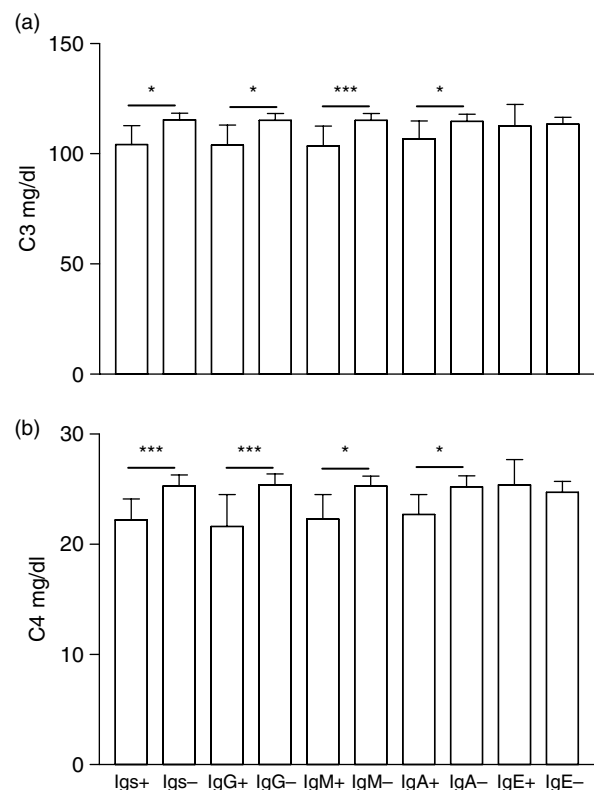


Fig. 1. (a) C3 and (b) C4 levels in sera with positive and negative anti-*Anisakis simplex* antibody levels $\geq X + SD$. Results are expressed as mean and confidence intervals of 95%. * $P < 0.05$, *** $P < 0.005$.

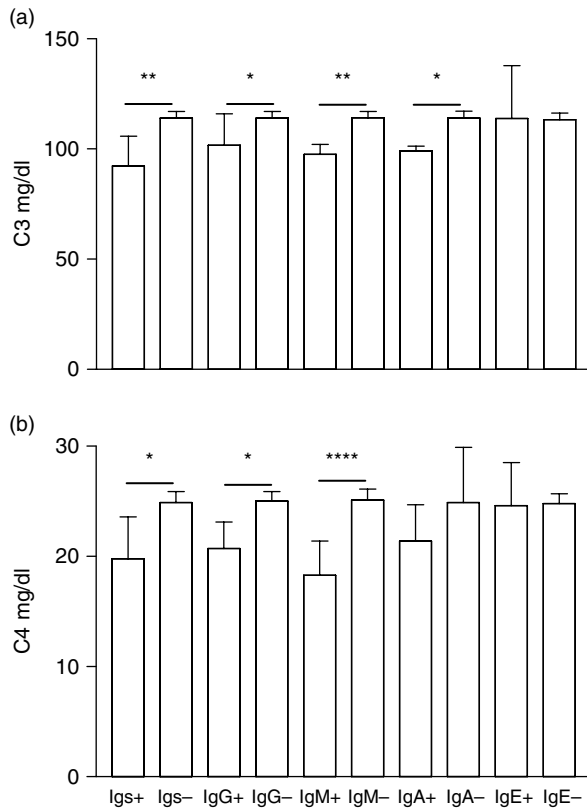


Fig. 2. (a) C3 and (b) C4 levels in sera with positive and negative anti-*Anisakis simplex* antibody levels $\geq \bar{X} + 2SD$. Results are expressed as mean and confidence intervals of 95%. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.001$.

Burlingame, California, USA) (Gutiérrez & Cuéllar, 2002; Martínez de Velasco & Cuéllar, 2003). Values higher than the mean ($\bar{X}$) of the optical density (OD) of 309 subjects obtained for each immunoglobulin and antigen plus once or twice their respective standard deviation (SD) were considered as positive in order to do the statistical treatment to compare the results with C3 and C4 levels.

Measurement of C3 and C4 complement

The quantitative measurement of C3 and C4 was performed using the turbidity assay from VITROS Chemistry Products (Ortho-Clinical Diagnostics, Inc.,

Rochester, New York, USA) according to the manufacturer's instructions.

Data analysis

The statistically significant relationship between specific anti-*A. simplex* antibody levels and serum levels of C3 and C4 were studied. The Student's *t*-test or Mann-Whitney *U* was used to compare means of variables. Contingence tables were drawn up and odds ratio (OR) and its 95% confidence intervals (CI) were calculated as a measure of association. The Pearson's test was used to study correlation. The level of significance was taken as $P < 0.05$.

Results and discussion

The main objective in our study was to measure levels of C3 and C4 in randomly selected human sera and to correlate these concentrations with the presence of anti-*A. simplex* specific antibodies. Normal values of C3 and C4 levels are 90–180 mg/dl and 10–40 mg/dl, respectively, according to the test used. In a first step, C3 and C4 levels were significantly lower in subjects who presented anti-*A. simplex* antibodies, with the exception of IgE (figs 1 and 2). Secondly, in order to compare the obtained results, values higher than the mean of the OD of the 309 subjects obtained for each anti-*A. simplex* immunoglobulin plus once or twice their respective standard deviations were considered as positive. Antibody prevalences expressed in percentages are shown in tables 1 and 2. Differences in C3 and C4 values with respect to anti-*A. simplex* negative samples were studied (tables 1, 2 and 3). The presence of an association between anti-*A. simplex* elevated levels and C3 deficiency (tables 1 and 2) has been demonstrated. The association between anti-*A. simplex* antibody levels and C4 deficiency was not significant because of the low number of subjects with C4 deficiency (table 3). By means of the Pearson test a negative correlation that was statistically significant between C3/C4 levels expressed in mg/dl and anti-*A. simplex* antibodies expressed in OD was observed, with the exception of IgE. The highest inverse correlation was obtained in the case of anti-*A. simplex* IgM.

In the case of immunoglobulins, the probability that a subject with anti-*A. simplex* antibodies has C3 deficiency was 3.8 times higher than a subject who lacks such specific antibodies (table 1). Likewise, although the

Table 1. Deficiency in C3 (<90 mg/dl) in sera with positive and negative anti-*Anisakis simplex* antibody levels $\geq \bar{X} + SD$.

Antibodies	Sera with anti- <i>A. simplex</i> N (%)	Sera with positive deficiency N (%)	Sera with negative deficiency N (%)	OR (CI 95%)	P
Igs	56/309 (18.1)	21/56 (37.5)	34/253 (13.4)	3.87 (2.02–7.40)	<0.001
IgG	51/309 (16.5)	18/51 (35.3)	37/258 (14.3)	3.26 (1.66–6.38)	0.001
IgM	51/309 (16.5)	17/51 (33.3)	38/258 (14.7)	2.90 (1.47–5.69)	0.004
IgA	54/309 (17.4)	15/54 (27.8)	40/255 (15.7)	2.07 (1.04–4.10)	0.049
IgE	26/309 (8.4)	5/26 (19.2)	50/283 (17.7)	1.11 (0.40–3.08)	0.792

Igs, immunoglobulins; N, number; OR, odds ratio; CI 95%: 95% confidence interval.

Table 2. Deficiency in C3 (<90 mg/dl) in sera with positive and negative anti-*Anisakis simplex* antibody levels $\geq X + 2SD$.

Antibodies	Sera with anti- <i>A. simplex</i> N (%)	Sera with positive deficiency N (%)	Sera with negative deficiency N (%)	OR (CI 95%)	P
Igs	12/309 (3.9)	7/12 (58.3)	48/297 (16.2)	7.26 (2.21–23.83)	0.002
IgG	17/309 (5.5)	7/17 (41.2)	48/292 (16.4)	3.56 (1.29–9.81)	0.018
IgM	17/309 (5.5)	7/17 (41.2)	48/292 (16.4)	3.56 (1.29–9.81)	0.018
IgA	16/309 (5.2)	7/16 (43.8)	48/293 (16.4)	3.97 (1.41–11.18)	0.012
IgE	8/309 (2.6)	2/8 (25.0)	53/301 (17.6)	1.56 (0.31–7.94)	0.636

Igs, immunoglobulins; N, number; OR, odds ratio; CI 95%, 95% confidence interval.

Table 3. Deficiency in C4 (<10 mg/dl) in sera with positive anti-*Anisakis simplex* antibody levels $\geq X + SD$.

Antibodies	Sera with anti- <i>A. simplex</i> N (%)	Sera with positive deficiency N (%)	Sera with negative deficiency N (%)	OR (CI 95%)	P
Igs	56/309 (18.1)	2/56 (3.6)	2/256 (0.8)	4.65 (0.64–33.63)	0.152
IgG	51/309 (16.5)	2/51 (3.9)	2/258 (0.8)	5.22 (0.72–33.98)	0.129
IgM	51/309 (16.5)	2/51 (3.9)	2/258 (0.8)	5.22 (0.72–33.98)	0.129
IgA	54/309 (17.4)	0/54 (0.0)	4/255 (1.6)	0.98 (0.97–1.00)	1.000
IgE	26/309 (8.4)	0/26 (0.0)	4/283 (1.4)	0.98 (0.97–1.00)	1.000

Igs, immunoglobulins; N, number; OR, odds ratio; CI 95%, 95% confidence interval.

number of C4-deficient individuals was low, C4 levels were lower in *A. simplex* positive subjects. The suggestion of the present investigation could be that a C4 deficiency increased susceptibility to *A. simplex* infection to a similar extent as a C3 deficiency and could support the hypothesis that the CP, and not the AP, is primarily involved in an antibody-independent humoral immunity to this parasite. The confirmation of these findings could also demonstrate the participation of the complement system in *A. simplex* third-stage larvae destruction as was demonstrated in *Angiostrongylus cantonensis* meningo-encephalitis (Padilla-Docal *et al.*, 2009). Leventhal & Soulsby (1977) observed that, with time, C3 levels remained markedly depressed (95% depletion) in animals treated with cobra venom factor (CVF), suggesting that the C3 depletion caused by CVF allowed increased eosinophilic infiltration in guinea-pigs infected with *Ascaris suum* larvae.

Increased C3 and C4 consumption has been demonstrated for other digestive pathogens; for example, Dore *et al.* (2005) observed lower levels of C3 and/or C4 complement fractions in *Helicobacter pylori*-infected subjects with respect to controls. On the other hand, Torcal *et al.* (1996) observed that patients with hydatidosis showed a significant increase of IgE and a decrease of C3 when cysts were large and multiple, and Leventhal & Soulsby (1977) demonstrated that the third component of complement can bind to the surface of *A. suum* larvae. The involvement of the complement system by these and other agents, such as *A. simplex*, should cause an increased consumption of complement components such as C3 and C4. In our study the lower concentration of C3 and C4 in sera with high anti-*A. simplex* antibody concentration can best be explained by an increased consumption of C3 and C4 components. *Anisakis simplex* may produce complement consumption and evade the

host immune system. Our previous work (García-Hernández *et al.*, 2007) suggested that the consumption of complement factors represents a mechanism of action of *A. simplex* larval products, since shortening of the preincubation period (0 and 15 min), compared with the standard time of 30 min, at 37°C did result in a significant decrease of the inhibitory effect on the lysis of *A. simplex* products in the CP.

According to Da Costa *et al.* (1999), the expression of complement inactivators by the parasite might represent an important protective mechanism to avoid host detection and induction of a vigorous IgG memory response. On the other hand, there is an important relationship between human parasite infection induced by *A. simplex* and urticaria (Daschner *et al.*, 2008) and hypocomplement urticarial vasculitis presents as atypical urticaria where hypocomplementaemia is the characteristic feature, with reduced C3 and C4 (Frot *et al.*, 2008).

In conclusion, the presence of an association between high levels of anti-*A. simplex* antibodies and C3/C4 deficiency has been proved. This study demonstrates serological evidence for the involvement of the complement system in the pathophysiology of human *A. simplex* infection. To our knowledge, this is the first study analysing specific immunoglobulin levels against larval antigens in this common fish-borne disease in relation to complement levels in humans. Additional studies are necessary to ascertain the clinical significance of these modifications.

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