

Positive associations between infections of *Toxoplasma gondii* and seropositivity with *Anisakis simplex* in human patients suffering from chronic urticaria

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Abstract

Toxoplasma gondii is a food-borne and orofecal microorganism which produces chronic infection, and attempts have been made to prove its negative association with atopy in the context of the hygiene hypothesis. *Anisakis simplex* is a fish parasite associated with chronic urticaria (CU) in endemic regions. We analysed the relationship between both infectious agents in CU. We included 42 patients with chronic urticaria (18 patients with CU associated with *A. simplex* sensitization and 24 not sensitized CU patients). Patients were assessed for atopy by a skin prick test (SPT) against common aeroallergens and for respiratory symptoms. *Anisakis simplex* sensitization was assessed by SPT and specific IgE by CAP fluoro-enzyme immunoassay (CAP-FEIA). Anti-*T. gondii* IgG levels were measured by enzyme-linked immunosorbent assay (ELISA). CU patients were analysed with respect to *T. gondii* seropositivity, *A. simplex* sensitization, atopy and immigrant status. The seroprevalence of *T. gondii* was 40.5% in CU patients and 42.1% in the control group. Immigrants were more frequently infected by *T. gondii* (41.2% versus 12%; $P = 0.036$). Anti-*T. gondii* IgG antibodies were associated with past *A. simplex* parasitism (odds ratio 6.73; $P = 0.03$) and independently with atopic sensitization (odds ratio 5.85; $P = 0.04$). In CU patients, *T. gondii* has no protective effect on atopic sensitization or *A. simplex* sensitization.

Introduction

In past decades a rise has been observed in the prevalence of allergic disease, most noteworthy in westernized populations (Bach, 2002; Okada *et al.*, 2010). Within the important role of environmental factors suggested as an explanation, the hygiene hypothesis

has received outstanding attention (Strachan, 1989). It has been postulated that diminished exposure to infectious or commensal agents in the first years of life could be associated with a higher risk of atopic disease, but also with autoimmune and chronic inflammatory disease, such as type 1 diabetes mellitus, inflammatory bowel disease or multiple sclerosis (Maizels, 2005; Okada *et al.*, 2010). Whereas initial disequilibrium in the balance of Th1/Th2-mediated immunologic response has been proposed to be responsible for the rise in allergic disease, epidemiological evidence demonstrated that Th1-associated

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inflammatory disease was also rising (Sheikh *et al.*, 2003). Thus, in recent years the revised hygiene hypothesis states a defect in regulatory features of an evolved immunologic system, which needs input from evolutionary co-evolved micro-organisms or parasites (Yazdanbakhsh & Matricardi, 2004; Rook, 2007).

Toxoplasma gondii is a cosmopolitan intracellular protozoan parasite, which affects more than 30% of the human population (Matowiczka-Karna *et al.*, 2009). Its prevalence depends on geography, age and eating habits, and is generally considered a marker of poor hygiene (Linneberg *et al.*, 2003). In immuno-competent hosts, infection by *T. gondii* is chronic and mainly asymptomatic. Several orofecally transmitted infectious agents have been associated with a diminished allergic response, but in the case of *T. gondii* this fact has not been currently demonstrated (Ellertsen *et al.*, 2008; Fenoy *et al.*, 2009).

Chronic urticaria (CU) is a common and disabling disease characterized by recurrent appearance of wheals. A recent epidemiological survey described a lifetime prevalence rate of CU of up to 1.8% and it has repeatedly been shown to affect quality of life (Zuberbier *et al.*, 2010). Whereas previous studies did not establish the association of CU with a polarized Th1 or Th2 milieu but demonstrated a mainly Th0 or mixed Th1/Th2 profile (Ferrer *et al.*, 2002), a recent study proposed that CU is associated with changes in the Th17/Treg axis (Daschner *et al.*, 2011). Further, an autoimmune basis can be observed in a high proportion of patients (Grattan *et al.*, 1991). The aetiology of CU is mainly unknown and has not been analysed regarding the hygiene hypothesis, but there is epidemiological evidence for an association of subgroups of CU with infectious agents, such as *Helicobacter pylori* or *Anisakis simplex* (Wedi *et al.*, 2004; Daschner *et al.*, 2010a).

As in our region a high proportion of CU is associated with *A. simplex* sensitization (Daschner & Pascual, 2005; Cuéllar *et al.*, 2010, 2012), we hypothesized that Th2-associated *A. simplex* sensitization and Th1-associated *T. gondii* infection could be negatively associated. Therefore we studied patients with CU with respect to these immunologically antagonistic infectious agents.

Materials and methods

Collection of serum samples

We included prospectively 42 patients with chronic urticaria (CU). Chronic urticaria patients were included if recurrent wheals were present at least twice weekly for a minimum of 6 weeks. Patients were not included in this study if physical stimuli were the main eliciting agents of the urticarial reaction. If patients displayed positive skin prick test (SPT) and specific IgE against *A. simplex*, they were included in the CU+ group. CU- patients showed neither a positive SPT nor detectable specific serum antibodies against *A. simplex*. Eighteen patients belonged to the *A. simplex* sensitization associated CU group (CU+) and 24 were not sensitized (CU-). Nineteen native subjects without a history of urticaria served as controls. Patients were assessed for atopy from SPT and for physician-diagnosed respiratory symptoms. Natives were compared to immigrants from tropical and

subtropical regions of South America. Written consent was obtained from all studied subjects.

Skin Prick Test (SPT)

The SPT was performed with *A. simplex* (Lab. ALK-Abelló, Madrid, Spain) and against the most frequent aeroallergens in our area: animal dander (cat, dog), house-dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*), pollen of *Cupressus arizonica*, *Olea europea*, *Lolium perenne*, weed mix and mould *Alternaria alternata* (Lab. ALK-Abelló). SPTs were performed by standard technique and considered positive with a mean wheal diameter of 3mm or more. Histamine at 1% concentration and saline solution 0.9% (NaCl) were positive and negative controls, respectively. Wheal diameter was measured 15 min after treatment.

Serology

Specific IgE against *A. simplex* and total IgE were assessed by CAP fluoro-enzyme immunoassay (CAP-FEIA; Phadia, Uppsala, Sweden). In order to reduce false-positive results, patients were only included in the *Anisakis* sensitization associated group if they displayed specific IgE >1.5 kU/l.

For the determination of *T. gondii* infection, specific IgG levels were measured by the NovaLisTM *Toxoplasma gondii* IgG enzyme-linked immunosorbent assay (ELISA) system (NovaTec Immundiagnostica GmbH, Dietzenbach, Germany) according to the test procedure. Briefly, serum samples were diluted at 1/100. In order to obtain quantitative results in IU/ml the (mean) absorbance values of the four Standards A, B, C and D were plotted on a standard calibration curve against their corresponding concentrations (0, 50, 100 and 200 IU/ml). According to WHO guidelines, sera with values ≥ 35 IU/ml were considered positive.

Data analysis

Statistical analysis was performed using SPSS version 15.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Prevalences were calculated for sex, atopy, respiratory symptoms, sensitization against *A. simplex* (comparing CU- and CU+) and immigrant status, and compared by chi-square test and odds ratio (OR) with 95% confidence interval (CI). Mean age was calculated in all studied groups and compared by ANOVA (normal distribution of data). For total and specific IgE, median and interquartile range (IQR) were calculated for and compared by Mann-Whitney analysis. A logistic regression model was performed, including variables that reached a significance level of $P < 0.05$.

Results

Mean age in all studied subjects was 43.2 ± 14.6 years and the proportion of women was 62.3%. According to atopic condition, 37.7% had physician-diagnosed rhinoconjunctivitis and/or bronchial asthma, and 60.7% had at least one positive SPT against aeroallergens.

When the origin of population was investigated, we observed that 16.4% were immigrants.

Median anti-*Anisakis* specific IgE in the CU+ group was 5.6 kU/l (IQR, 2.1–12.3 kU/l). Expectedly, total IgE serum levels were also higher in the CU+ group (median, 240; IQR, 60.8–601 kU/l) than in the CU– group (median, 103.5; IQR, 36.5–157 kU/l; $P = 0.03$). The prevalence of *T. gondii* infection, measured as specific IgG levels ≥ 35 IU/ml by ELISA, was 42.1% in the control population and 40.5% in all CU patients (not significant). There was a higher prevalence of *T. gondii* infection in the CU+ than in the CU– group; however, the difference did not reach statistical significance in this bivariate analysis ($P = 0.08$; fig. 1). Patients with IgG anti-*T. gondii* levels ≥ 35 IU/ml had a higher prevalence of sensitization against aeroallergens than seronegative patients (82.4% versus 44%, $P = 0.014$; fig. 2). Also these patients were mainly sensitized against pollen (58.8% versus 32%, $P = 0.08$), and less against house-dust mites, mould or animal dander, when compared with the *T. gondii*-negative group. The prevalence of respiratory symptoms did not differ between the studied groups. Patients with CU and *T. gondii* infection were more likely to be immigrants (41.2%) than non-infected CU patients (12%, $P = 0.036$; fig. 3).

Immigrants in our region are mainly from South America and probably do not have the same fish-eating habits as natives. Since frequent contact with larvae of *A. simplex* could be less likely in these subjects, a logistic regression model was performed in order to analyse the relationship between *A. simplex* sensitization, immigrant status and atopy. In this analysis *A. simplex* sensitization as well as atopy reached statistical significance in explaining a higher probability to be also infected with *T. gondii*, and the significance of a positive association between immigrant status and *T. gondii* maintained only a near significant level (table 1). Although, according to Flegr & Striž (2011), women had the highest probability of *T. gondii* infection, in our study there were no significant differences regarding sex between both seropositive and seronegative groups. For this reason, this variable was not included in the logistic analysis as an independent variable.

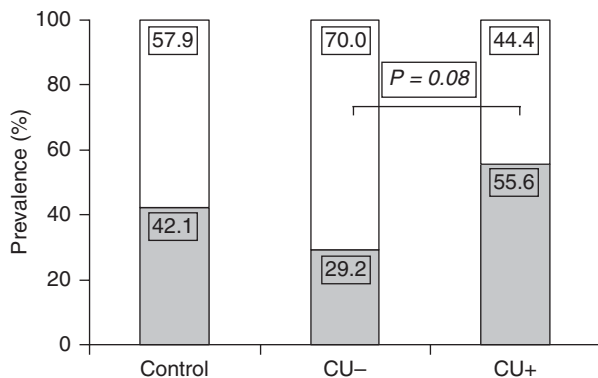


Fig. 1. The prevalence (%) of *Toxoplasma gondii* infection assessed by ELISA; *Anisakis simplex* sensitization associated with chronic urticaria (CU+) and without sensitization (CU–), positive (grey), negative (white).

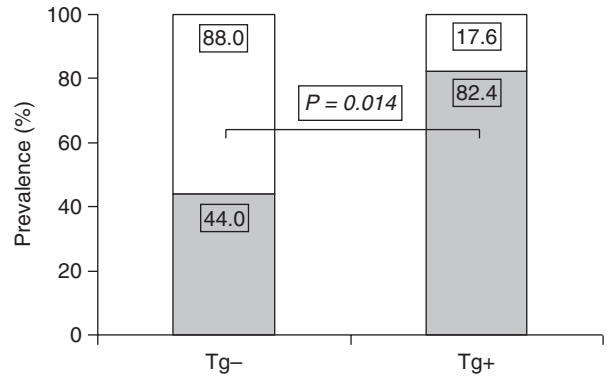


Fig. 2. The prevalence (%) of atopy relative to *Toxoplasma gondii* infection in patients with chronic urticaria and independent of *Anisakis simplex* sensitization; no *T. gondii* infection (Tg–), seropositivity with *T. gondii* infection (Tg+), positive (grey), negative (white).

Discussion

The data of seropositivity as assessed by anti-*T. gondii* specific IgG determination by ELISA were in accordance with those obtained in previous studies carried out in a European population (50–80%) (Hill & Dubey, 2002). The comparison of the prevalence of *T. gondii* infection showed no significant differences between CU patients and controls. When the prevalence of IgG anti-*T. gondii* was analysed with respect to immigrant status, 70% seropositivity was obtained. This percentage was much higher than the 40.5% obtained in the total group of CU patients. It should be noted that 23.8% of CU patients of the study were Latino-American immigrants. This high prevalence of IgG anti-*T. gondii* observed in our study is similar to that reported by others in immigrants from tropical countries and is consistent with known higher prevalence rates in regions with 'poorer' hygiene. Previous studies have provided significant data on the high seroprevalence of *T. gondii* in Central and South America (Montoya & Liesenfeld, 2004; Ramos *et al.*, 2011). Likewise, Ramos *et al.* (2011) found 41.1% of *T. gondii*-positives in a study conducted on pregnant immigrants in Spain, compared to 12% of positives in Spanish women. Thus, the *Toxoplasma* seropositivity data in our CU patients parallel previously known data in the general population.

However, in the study group we detected an unexpected positive association of chronic infection by *T. gondii* measured by IgG determination and specific IgE against *A. simplex* as a marker of previous parasitism by this nematode. A synergism between both parasites was observed since 58.82% of *T. gondii*-positive sera were *A. simplex* positive. Thus the likelihood of chronic urticaria is enhanced when specific antibodies against *A. simplex* and *T. gondii* occur simultaneously. *Anisakis simplex* is a cosmopolitan fish parasite which is able to produce allergic reactions when fish is consumed raw or undercooked (López-Serrano *et al.*, 2000; Alonso-Gómez *et al.*, 2004). Whereas gastro-allergic anisakiosis (GAA) is accompanied by acute urticaria, angioedema or

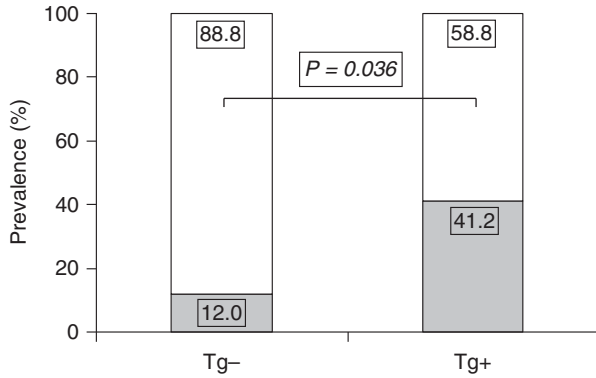


Fig. 3. The prevalence (%) of *Toxoplasma gondii* infection in South American immigrants with chronic urticaria showing seropositivity (Tg+) or no *T. gondii* (Tg-); immigrants (grey), non-immigrants (white).

anaphylaxis in the context of an acute parasitism, in *A. simplex* associated CU (CU+), the moment of parasitism can not be elucidated by clinical history (Daschner *et al.*, 2000, 2010b). In any case, a long-term parasitism by this nematode is not possible in humans, as the third-stage larva is not able to survive in the human host for more than a few days. Nevertheless, even in short-lived parasitism as in GAA, where the larva is rapidly expelled, the immunological response produces an important elevation of specific and total IgE and the SPT remains afterwards positive in all forms of human parasitism (Daschner *et al.*, 2001). This demonstrates the typical Th2-driven immunological feature elicited by helminth parasites, to which *A. simplex* belongs. However, *T. gondii* is associated with a Th1-driven immunological response before it produces chronic infection. Thus, our data show that known Th1 response by *T. gondii* infection is not able to inhibit the Th2 response by *A. simplex*. For the establishment of the chronic phase of the infection, *T. gondii* induces the secretion of leukotriene A4, the potent anti-inflammatory action of which decreases interleukin (IL)-12 production (Aliberti *et al.*, 2002). These low levels of IL-12 could result in increased Th2 cytokines and, therefore, an increased presence of allergic reactions (Yazdanbakhsh & Matricardi, 2004), as occurs after infection by *A. simplex*. However, the Th2 response by *A. simplex* is an evolutionarily conserved mechanism that cannot be regarded as an atopy marker (Daschner & Cuéllar, 2010).

In fact, it is not to be expected that different environments would prevent infectious agents belonging to one Th1 or Th2 associated immune response, to prevent the host from mounting a sufficiently effective immune response to organisms belonging to the other immune response type. Janson *et al.* (2007) found no association between levels of IgG antibodies to *T. gondii*, *H. pylori* and hepatitis A virus, and total IgE levels in a group of subjects from Iceland, Estonia and Sweden. Similarly, Fernandes *et al.* (2010) found no significant differences in total IgE levels measured in *T. gondii*-positive and *T. gondii*-negative subjects, although the total IgE levels were significantly higher in atopic individuals

of both *T. gondii*-tested groups. Matricardi *et al.* (2000) found no differences in total IgE levels among *T. gondii*-positive and *T. gondii*-negative subjects, but different levels of specific IgE against different inhalant allergens were observed. Consequently, they proposed that total IgE levels are controlled by different regulatory mechanisms than those that are regulating the levels of specific IgE. However, our results are in accordance with those of Seiskari *et al.* (2007), who found a positive association between total IgE levels and positivity to *T. gondii* in patients from Finland and Russia. This correlation could be a consequence of the deviation of the immune response towards a Th2 response, resulting from a reduction of the Th1 response, which occurs during the chronic phase of toxoplasmosis. In spite of this immunological explanation, the association of both *Toxoplasma* and *Anisakis* parasites is related to environmental factors and dietary habits. Supporting this hypothesis, Jones *et al.* (2008) postulated an association of anti-*T. gondii* IgG with anti-*Toxocara* IgG antibodies by a common soil-transmitted infection route. In this case, *T. gondii* infection was not able to inhibit atopy and, interestingly, our results showed an association between atopy with *T. gondii* infection. In addition to the previously reported low IL-12 levels in chronic *T. gondii* infection (Yazdanbakhsh & Matricardi, 2004), Perona-Wright *et al.* (2009) identified the same effect induced by natural killer (NK) cells that rapidly express IL-10 during acute toxoplasmosis.

Two arguments highlight the straightforward positive association of atopy and *T. gondii* infection in CU patients. The multivariate analysis demonstrates that atopy and *A. simplex* sensitization are both independent variables explaining risk of *T. gondii* infection. Further, even if *A. simplex* sensitization has previously been associated with house-dust mite (HDM) sensitization (Daschner *et al.*, 2008), in the present study HDM sensitization did not account for the atopic sensitization, which was due mainly to pollen sensitization (data not shown). We observed a positive association close to significance between positivity to *T. gondii* and pollen sensitization. On the other hand, positivity to *T. gondii* has been reported to reduce allergic sensitization against the major allergen of birch pollen (*Bet v 1*), determined by the levels of specific IgE to *Bet v 1* in BALB/c mice experimentally infected before sensitization (Wagner *et al.*, 2009). Our results suggest that the presence of IgG anti-*T. gondii* is a risk factor for atopy in patients with chronic urticaria. When analysing with respect to the hygiene hypothesis, our results would render both analysed organisms as associated with 'poor' hygiene, thus conferring *A. simplex*,

Table 1. Variables explaining *Toxoplasma gondii* infection in a logistic regression model.

	Odds ratio	P	Standard error
<i>A. simplex</i> sensitization	6.73	0.03	0.87
Atopy	5.85	0.04	0.87
Immigrant status	0.18	0.08	0.97
Constant	0.33	0.32	1.1

like *T. gondii*, a possible anti-inflammatory role, consistent with the better prognosis of sensitized versus non-sensitized CU patients (Daschner & Pascual, 2005). On the other hand, whereas helminth parasitism has mainly been associated with protection against atopy in developing countries, *A. simplex* only produces acute parasitism and is not able to produce sufficient regulatory features, an equivalent to sporadic parasitism, proposed to be positively associated with atopy (Yazdanbakhsh & Matricardi, 2004).

The study protocol was not designed to assess the described associations in the general population, so we can not generalize our results and have to interpret them in the context of our studied patients. There is not sufficient knowledge of immunological mechanisms associated with CU in order to characterize this entity as a well-defined immunological abnormality. CU has not been associated with a Th1 or a Th2 phenotype, and in the present study, as a whole group, *T. gondii* seropositivity was similar to that of our control group. Higher pro-inflammatory cytokines such as IL-6 and tumour necrosis factor (TNF)- α have been associated with disease severity (Dos Santos *et al.*, 2008). A possible dysregulation in the Th17/Treg axis rather than the Th1/Th2 axis has also been proposed (Daschner *et al.*, 2011). Thus, we can not rule out that the, up to now poorly characterized, immunological features of CU are associated with both a Th1 and Th2 response against infectious agents.

Toxoplasma gondii belongs, together with hepatitis A virus and *H. pylori*, to the food-borne, orofecal micro-organisms, which have been associated with a lower prevalence of atopy (Ellertsen *et al.*, 2008). In contrast, Bodner *et al.* (2000), Linneberg *et al.* (2003) and Janson *et al.* (2007) observed no differences between the percentages of *T. gondii*-positive individuals in groups of patients diagnosed as atopic and non-atopic in studies in Iceland, Estonia, Sweden, Denmark and Scotland. The opposite finding in our studied group gains special interest, as *H. pylori* is one of the infectious agents for which a possible aetiological role has emerged in the study of CU (Wedi *et al.*, 1998). Thus, future studies should focus not only on the possible positive association of infectious agents with CU, but also on the possible different prognosis (mainly severity and duration) and its relationship with distinct pro- or anti-inflammatory cytokine production (Bermudez *et al.*, 1993). We previously compared pro- and anti-inflammatory as well as Th1- and Th2-associated serum cytokine levels in CU+ and CU- patients. Although serum levels of IL-10 and interferon (IFN)- γ were lower in CU+ and CU- subjects with respect to the control group, CU+ patients displayed higher transforming growth factor (TGF)- β levels than CU- subjects and controls (Daschner *et al.*, 2011). Furthermore, cytokine production of peripheral blood mononuclear cells from CU+ and CU- patients was studied, observing that after stimulation with *A. simplex* extract IL-2, IL-4 and IFN- γ production was higher in CU+, while Concanavalin A-induced IL-6 and IL-10 production was also higher in CU+ (Daschner *et al.*, 2013). Both studies demonstrated that different clinical and immunological phenotypes of urticaria are associated with distinct cytokine levels. For this reason the role of cytokines in protection against atopy in the course

of *T. gondii* infection or *A. simplex* parasitism need to be clarified. In conclusion, our data show in CU a positive and independent association of *T. gondii* infection with past *A. simplex* parasitism as well as with an atopic status.

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Conflict of interest

None.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The project was approved by the Ethics Committee of the University Hospital de la Princesa, Madrid.

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