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Short communication

Cross-reactivity between *Anisakis simplex* sensitization and visceral larva migrans by *Toxocara canis*

M.J. Perteguer [a], C. Cuéllar [a,*], J.L. Guillén [a], C. Águila [b], S. Fenoy [b], T. Chivato [c], R. Laguna [c]

[a] *Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain*
[b] *Sección de Parasitología, Facultad de Ciencias Experimentales y Técnicas, Universidad San Pablo, Madrid, Spain*
[c] *Servicio de Alergia e Inmunología Clínica, Hospital Universitario del Aire, Madrid, Spain*

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Abstract

The aim of this work was to study cross-reactivity in the diagnosis of two related ascaridosis. Nineteen patients diagnosed with recidivous acute urticaria (RAU) caused by *Anisakis simplex* and 26 patients diagnosed with visceral larva migrans (VLM) caused by *Toxocara canis* were studied employing commercial diagnostic kits and "in house" assay kits. Cross-reactivity observed was greater when using "in house" assay kits, suggesting that *T. canis* excretory–secretory antigens were not only recognized by antibodies from patients with RAU but with greater intensity compared to the *A. simplex* excretory–secretory antigens.
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Anisakis simplex and *Toxocara canis* are two nematodes commonly involved in human parasitism producing what are known as anisakiosis and the visceral larva migrans (VLM) clinical syndrome, respectively. Gastric anisakiosis can be readily diagnosed by detecting the worm using gastrofiberscopic examination. However, clinical diagnosis of intestinal anisakiosis is relatively difficult and the worm is occasionally found during a histopathological examination of the intestinal submucosa resected from the ileum (Ishikura et al., 1993). Due to the vagueness of its symptoms, this disease is often misdiagnosed (Sakanari and Mckerrow, 1989). Serodiagnostic tests based on a variety of techniques, have been designed (Akao an Yoshimura, 1989; Petithory et al., 1986; Sakanar et al., 1988; Tsuji, 1989). The sensitivity of serological methods have been improved due to the use of ELISA techniques (Poggensee et al., 1989); however, their specificity is still limited as a result of cross-reactivity (Iglesias et al., 1996).

Visceral larva migrans (VLM) is a clinical syndrome caused by the migration of *Toxocara canis* larvae through the extra-intestinal tissues of man (Beaver 1969; Glickman et al., 1979). The criteria used for the clinical diagnosis of human toxocariasis were selected by Barriga (1988), who grouped them in diminishing order according to their diagnostic significance (peripheral eosinophilia, leukocytosis, hypergammaglobulinaemia, hyperisohaemagglutinaemia, anemia,

* Corresponding author. Fax: +34-91-394-1815.
E-mail address: cuellarh@farm.ucm.es (C. Cuéllar).

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hypoalbuminaemia, hepathomegaly, fever, malnutrition, splenomegaly, anorexia and respiratory, digestive and nervous disturbances). The lack of eggs or larval stages in the faeces, together with the limited possibilities of histological techniques, has encouraged the development of immuno-diagnostic techniques. Evaluation of these techniques has led to the choice of ELISA with the larval ES antigen for the diagnosis of human toxocariasis (De Savigny, 1975; Cypess et al. 1977; Glickman et al., 1978).

There is no doubt about the existence of antigenic cross-reactivity between related nematodes and their consequences for immunodiagnosis. Several investigations have studied the possible existence of cross-reactions among different ascarids (Smith et al., 1982; Olson, 1960; Richards et al., 1962; Stevenson and Jacobs, 1977; Cypess et al., 1977; Grelk et al., 1981; Smith et al. 1983a,b).

In other studies, larval ES products and homogenized *T. canis, Toxocara leonina* and *Anisakis suum* adult worms extract were used by us to assess the possible cross-reactions in antisera raised in BALB/c and C57BL/10 mice inoculated or immunized with embryonated eggs or adult worm extract of *T. leonina* or *A. suum* in single and multiple doses (Cuéllar et al., 1992, 1995). The results obtained confirmed the specificity of the ES *T. canis* antigen which is routinely used in most immunological tests.

In the case of the nematode *A. simplex*, Iglesias et al. (1996) investigated the antigenic cross-reactivity in mice between third-stage larvae (L3) of *A. simplex* and the ascaridoids *A. suum* and *T. canis*. Their results indicated high antigenic cross-reactivity between *A. simplex* and the other ascaridoid nematodes. Yahiro et al. (1998) cloned the cDNA of TBA-1, the nematode polyprotein allergen (NPA) of *T. canis* and found it to be similar to ABA-1, the *Ascaris* NPA, on the basis of its amino acid sequence. They observed that the sera of rodents infected with *T. canis* larva or immunized with a *A. simplex* L3 larval extract recognized a different form of TBA-1 from rodents immunized with a *Toxocara* spp. adult worm. The authors suggested that two in vivo forms of TBA-1 are expressed but during different stages of the life cycle of the parasite. Romaris et al. (2001) by means of two monoclonal antibodies that recognize phosphorylcholine-bearing antigens, observed close similarities among the ascarids (*T. canis*, *A. simplex* and *A. lumbricoides*) suggesting that phosphorylcholine is attached to identical or very similar structures on many different nematode species.

In this work, we have attempted to verify whether, under our experimental conditions, cross-reactivity between *T. canis* and *A. simplex* appears. Thus, patients diagnosed with recidivous acute urticaria (RAU) caused by *A. simplex* and visceral larva migrans caused by *T. canis* were investigated.

To obtain the excretory–secretory (ES) antigen from *T. canis* and *A. simplex*, the larvae were cultivated in Minimum Essential Eagle Medium with Earle's salts (ICN Biomedicals, Inc., Costa Mesa, CA) supplemented with sodium bicarbonate (2.2 g/l), glutamine (0.292 g/l) and gentamicin (4 g/l) and maintained at 37 or 28 °C, respectively (Cuéllar et al., 1992; Perteguer et al., 1996). For the preparation of the *A. simplex* crude extracts (CE), L3 were homogenized, followed by sonication. The homogenate was extracted in PBS at 4 °C overnight, subsequently delipidized with *n*-hexane and then centrifuged (Perteguer et al., 1996). Nineteen patients with recidivous acute urticaria (RAU) and specific IgE to *A. simplex* were selected (Montoro et al., 1997). Patients diagnosed with any other allergic disease were excluded, as were those patients being treated with corticosteroids or antihistaminics. Twenty-six sera samples from patients with clinical characteristics of toxocariasis were selected after testing their antibody levels by means of ELISA using larval ES antigen from *T. canis* (Fenoy et al., 1997). *A. simplex* extract (International Pharmaceutical Immunology from ASAC, Pharmaceutical International, AIE) was tested on the RAU patients using the prick method. The concentration of circulating anti-*Anisakis* or *Toxocara* specific IgE was measured by means of Pharmacia CAP System RAST® FEIA (Pharmacia AB, Uppsala, Sweden), according to the instructions. Values of 0.35 kU/l of IgE and above represent a positive result. The results were also evaluated as RAST classes according to the instructions for the calculation of results.

We also used "in house" *Anisakis* and *Toxocara* ELISA diagnostic kits, and ES and/or crude extract of both parasites were tested. The 96-well microtitre plates (Nunc-Immuno Plate Maxi Sorp™) were coated with 0.1 μg per well of larval ES or CE antigen. Blocking was carried out by adding 0.1% BSA. Serum samples diluted 1/100 were

added in duplicate. Goat affinity isolated, horseradish peroxidase-conjugated anti-human immunoglobulins (γ-, α-, μ-, and light-chains), IgG (γ) and IgM (μ) (Caltag Laboratories, San Francisco, CA), were added. Substrate (*o*-phenylene-diamine, Sigma) was added with hydrogen peroxide. The reaction was stopped with 3N sulfuric acid and the plates were read at 492 nm (Montoro et al., 1997). For the evaluation of the results, we used the diagnostic index (DI) as the ratio between the OD resulting from the test serum and the OD of the negative control.

We examined a total of 19 patients with recidivous acute urticaria (RAU) due to *A. simplex*. These patients developed urticaria after ingestion of fish and/or other seafood. The *Anisakis* skin prick test was positive in 15 patients (Fig. 1(A)). In all the RAU cases, specific IgE to *A. simplex* with the Pharmacia CAP System RAST® FEIA was detected (mean: 9.2 ± 16.7 kU/l; minimum: 0.74; maximum; 71.4). No patient reacted to the *Toxocara* antigen using the same test (Fig. 1(A)). This absence of reaction was surprising because of the significant antigenic similarity demonstrated by Kennedy et al. (1988, 1989). These facts indicate that after infection with *T. canis* embryonated eggs cross-reactions are not generated against *A. simplex* somatic products. When specific immunoglobulin levels against *T.*

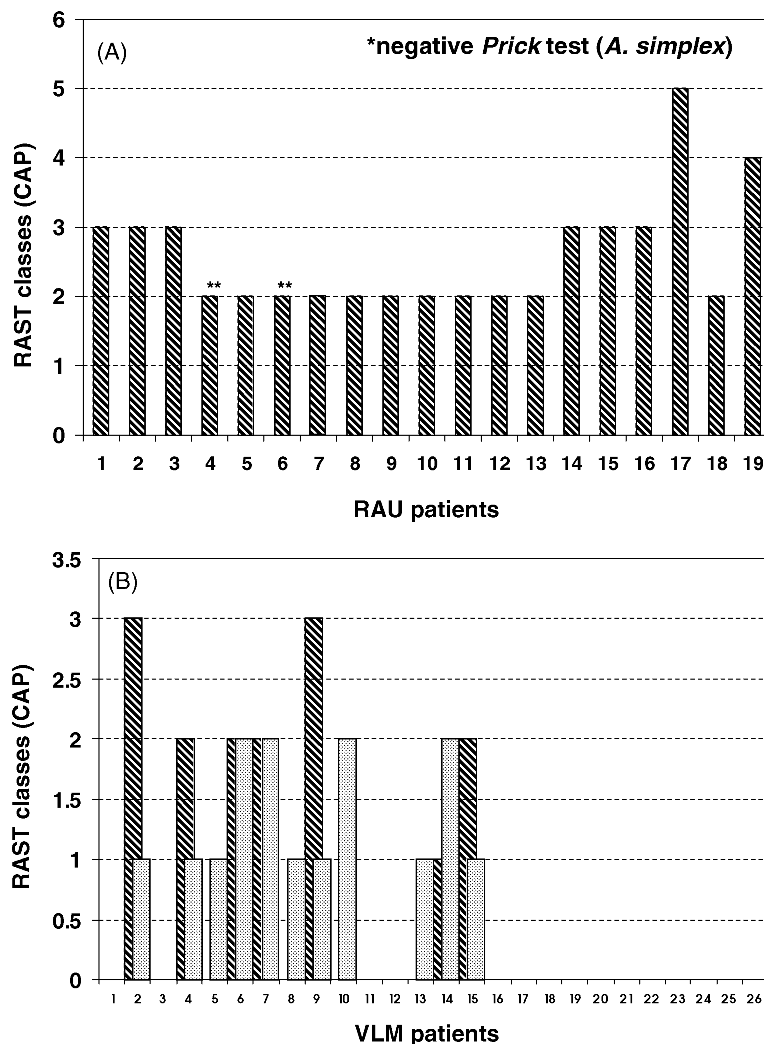

Fig. 1. Determination of anti-*Anisakis* (▧) and anti-*Toxocara* (▩) specific IgE by CAP system in sera from patients diagnosed of: (Panel A) recidivous acute urticaria (RAU) or (Panel B) visceral larva migrans (VLM). CAP = 0 (<0.35 kU/l); CAP = 1 (0.35–0.7 kU/l); CAP = 2 (0.7–3.5 kU/l); CAP = 3 (3.5–17.5 kU/l); CAP = 4 (17.5–50 kU/l); CAP = 5 (50–100 kU/l); CAP = 6 (>100 kU/l).

canis ES products using an "in house" ELISA were tested, 11 of 19 sera from RAU patients were found with DI over 3 which is considered positive (Fenoy et al., 1997). On the other hand, when these sera were tested by ELISA, the DI obtained against the *T. canis* ES antigen were higher than those observed in the *A. simplex* one.

Because of the greater cross-reactivity observed with sera from patients with RAU, we investigated if cross-reactions appeared by testing sera from patients diagnosed of VLM using *A. simplex* antigens. To carry out this experiment, specific IgE to *Anisakis* or to *Toxocara* were determined by the Pharmacia CAP System RAST® FEIA in sera from 26 patients with clinical diagnosis of VLM parasitic disease. Specific IgE to *Toxocara* was detected in 11 of the 26 patients (Fig. 1(B)). Only four VLM patients showed CAP = 2 (moderate according to Pharmacia CAP System RAST® FEIA). However, six patients showed specific IgE to *Anisakis* with CAP values $\geq$2.

The high levels of specific IgE to *A. simplex* obtained in our study, led us to investigate the levels of other isotype immunoglobulins. Total immunoglobulin IgG and IgM levels were tested against *A. simplex* CE, as were total immunoglobulins to ES products using sera from patients with a clinical suspicion of VLM (DI over 3 when tested by ELISA against *T. canis* ES antigen). Eighteen of 26 sera showed DI $>$ 2 of total immunoglobulins to *A. simplex* CE antigen and 23 and 11 sera, respectively, in the case of specific IgG and IgM levels. Twenty of the 26 VLM patients showed DI $>$ 2 of total immunoglobulins to *A. simplex* ES products.

T. canis ES antigen seems to be a major component in respect to *A. simplex* because such an antigenic preparation is always recognized with a higher intensity, both by antibodies from patients with clinical suspicion of VLM and by sera from patients diagnosed with RAU by *A. simplex*. In the latter group of patients, the responses were lower against their homologous ES antigen. This higher overbalancing of such antigens within the *T. canis* ES products is confirmed, inclusively, taking into consideration that *T. canis* larvae stay alive inside their hosts. Thus, the immune system is continually stimulated. On the other hand, after *A. simplex* infection, ES products are only produced in an early stage before the worm dies. Likewise, the antibody levels obtained with sera from patients diagnosed with RAU against *A. simplex* ES antigen were lower than those obtained testing sera from patients diagnosed with VLM against its homologous *T. canis* ES antigen.

The above fact should be considered in patients clinically diagnosed with helminth-associated asthma and serologically confirmed by detection of anti-*Anisakis* antibodies without a study of the anti-*Toxocara* antibodies (Estrada and Gozalo, 1997; Armentia et al., 1998). *Anisakis* might be able to induce asthma but it could also be useful in determining specific responses to *T. canis* ES in order to improve a differential diagnosis.

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