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Cross-reactivity induced by *Anisakis simplex* and *Toxocara canis* in mice

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Abstract

The aim of this study was to verify whether cross-reactivity appeared between *Toxocara canis* and *Anisakis simplex* in an experimental rodent model. No cross-reactions were detected using sera from mice infected with *T. canis* eggs. When responses obtained against *T. canis* ES antigen using sera from BALB/c and C57BL/10 mice infected with *T. canis* eggs were compared with those obtained by testing sera from mice infected with one *A. simplex* L3, an increase in cross-reactions was observed using the C57BL/10 strain.

Introduction

The possible existence of cross-reactions among different ascarids compromises the usefulness of most tests in the diagnosis of such parasitic diseases. For this reason, human and experimental cross-reactions have been the object of several studies. Human cross-reactions have been noted by several authors. Olson (1960), using the *in vitro* larval precipitate test and sera from patients, obtained one case with a recent history of *Ascaris lumbricoides* positive with *Toxocara* larvae as a source of antigen. Richards *et al.* (1962), employing the same test, observed that one patient infected with *Ascaris* had serum weakly positive to *Toxocara*. Stevenson & Jacobs (1977) observed cross-reactions with the immunofluorescent antibody (IFA) test, Cypess *et al.* (1977) using immunodiffusion, Grelk *et al.* (1981), by the enzyme linked immunosorbent assay (ELISA), and Smith *et al.* (1983a; 1983b) by immunoelectrophoresis, immunodiffusion and IFA tests.

Serological cross-reactions between different ascarids by means of experimental infections have been investigated by Cuéllar *et al.* (1992, 1995). Larval excretory–secretory (ES) products and homogenized *Toxocara canis*, *Toxascaris leonina* and *Ascaris suum* adult worm extracts were used 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. These results confirmed the specificity of the ES *T. canis* antigen, which is conventionally used in the diagnosis of human visceral larva migrans. In recent years, several cases of human anisakiasis, a related nematode disease, have been described demonstrating that the specifity of the diagnosis test in human toxocariasis and anisakiasis is not yet 100% specific.

The antigenic cross-reactivity in mice between third-stage larvae (L3) of *A. simplex* and the ascaridoids *A. suum* and *T. canis* was investigated by Iglesias *et al.* (1996) who found a high antigenic cross-reactivity between *A. simplex* and the remaining 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 most similar to ABA-1, the *Ascaris* NPA, on the basis of the amino acid sequence. These workers observed that rodents infected with *T. canis* larva or immunized with an *A. simplex* L3 larval extract reacted to this polyprotein differently from those immunized with *Toxocara* adult extract. In the present work we wanted to verify whether, in our experimental conditions, cross-reactivity between *T. canis* and *A. simplex* appears with the antigens used in the most common methods employed today in clinical studies, that is larval ES products of *T. canis* L2, larval ES products of *A. simplex* L3 and *A. simplex* L3 larval extract.

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Materials and methods

Parasites

Toxocara canis eggs were obtained by dissecting gravid females (Cuéllar *et al.*, 1992). *Anisakis simplex* third-stage larvae (L3) were obtained following the dissection of infected blue whiting (*Micromesistius poutassou*) (Perteguer & Cuéllar, 1998).

Antigens

Larvae of *T. canis* were obtained by hatching embryonated eggs in a mixture of equal parts of sodium hypochloride and sodium hydroxide at 2%. To obtain 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, California), supplemented with sodium bicarbonate ($2.2\,g\,l^{-1}$), glutamine ($0.292\,g\,l^{-1}$) and gentamicin ($4\,g\,l^{-1}$) and maintained at 37°C or 28°C, respectively (Cuéllar *et al.*, 1992; Perteguer *et al.*, 1996). To prepare the *A. simplex* crude extracts (CE), L3 were homogenized in a hand-operated glass tissue grinder at 4°C, followed by sonication for 10 s with a Virsonic 5 (Virtis) set at 70% output power. The homogenate was extracted in PBS at 4°C overnight and subsequently delipidized with n-hexane and then centrifuged (Biofuge 17RS, Heraeus Sepatech) (Perteguer *et al.*, 1996).

Experimental mice

Forty BALB/c mice were divided into four equal groups: group 1 (inoculation with a single dose with *T. canis* eggs); group 2 (inoculation with one L3 of *A. simplex*); group 3 (immunization with a single dose with CE *A. simplex* antigen); group 4 (immunization with multiple doses with CE *A. simplex* antigen). The same procedure was followed using C57BL/10 mice.

Inoculation protocol

Group 1 mice were inoculated with embryonated *T. canis* eggs as a single dose of 1000 embryonated eggs by oral administration with gastric tubing. Group 2 mice were inoculated with one L3 of *A. simplex* per animal by oral administration with gastric tubing. Group 3 mice were injected with CE *A. simplex* antigen ($1\,mg\,ml^{-1}$ in final volume) in Freund Complete Adjuvant (FCA) by the intramuscular route. Group 4 mice were injected with a single dose of 1 ml of CE antigen in FCA followed by multiple doses of 1 ml of CE antigen in Freund Incomplete Adjuvant (FIA) ($1\,mg\,ml^{-1}$ in final volume) given weekly for 2 weeks.

*ELISA for the determination of anti-*Toxocara *or anti-*Anisakis *specific of cross-reacting antibody levels*

The 96-well microtitre plates (Nunc-Immuno Plate Maxi Sorp™) were coated by the addition of $1\,\mu g\,ml^{-1}$ of larval ES or CE antigens. Blocking was carried out by adding 200 μl per well of 0.1% BSA in PBS. One hundred microlitres of serum samples diluted 1/100 were added in duplicate. One hundred microlitres per well of goat affinity isolated, horseradish peroxidase conjugated specific to mouse IgG + IgM (H + L), IgM (μ), IgG (γ), IgG1(γ1), IgG2a (γ2a) and IgG2b (γ2b) (Caltag Laboratories, San Francisco, California), were added to each well. One hundred microlitres per well of substrate (o-phenylene-diamine; Sigma) were added to each well at 0.04% with 0.04% hydrogen peroxide. The reaction was stopped with 3 N sulphuric acid and the plates were read at 492 nm. Results were expressed as O.D.p–O.D.c indexes by subtracting the mean optical density (O.D.) of control from the mean O.D. of test sera once the non-specific reaction with the BSA used in the blocking was subtracted.

Results and Discussion

Human cross-reactions between visceral larva migrans and anisakiasis have been broadly demonstrated. In the present study, an attempt has been made to verify whether, under experimental conditions, cross-reactivity between *T. canis* and *A. simplex* appears with the antigens currently used in clinical methodology, that is, larval ES products of *T. canis* L2, larval ES products of *A. simplex* L3 and *A. simplex* L3 larval extract. BALB/c and C57BL/10 mice strains were used because of their different humoral responses following *A. simplex* infection (Perteguer & Cuéllar, 1998), thereby allowing useful information to be obtained from these models.

In the first group of experiments, humoral responses against the crude extract (CE) of *A. simplex* third-stage larva (L3) were studied. BALB/c and C57BL/10 mice were orally inoculated with *T. canis* eggs, which resembled a human infection, and their responses were compared to those obtained with the following groups. In BALB/c and C57BL/10 mice immunized with CE *A. simplex* antigen in single and multiple doses, no cross-reactions were observed during the whole experiment, so data are not shown. In BALB/c and C57BL/10 mice inoculated with one *A. simplex* L3 by oral administration, which resembled human infection, no cross-reactions were detected when the responses obtained with sera from these animals were compared with those observed using sera from mice infected with *T. canis* embryonated eggs. Data are not shown.

When the humoral responses obtained against *T. canis* ES antigen were compared (fig. 1), cross-reactions were observed to be higher using sera from BALB/c and C57BL/10 mice infected with *T. canis* embryonated eggs than those obtained by testing sera from mice infected with one *A. simplex* L3. This result, apart from the previous results, indicates that following murine infection with *T. canis* embryonated eggs, cross-reactions are not generated in response to *A. simplex* somatic products. Conversely, cross-reactions were present when mice were infected with *A. simplex* and their sera were tested against ES *T. canis* antigen.

These results are in accord with those found by other authors (Yahiro *et al.*, 1998) in which sera from mice immunized with a crude extract elicit a different response from those inoculated with ES products in relation to their

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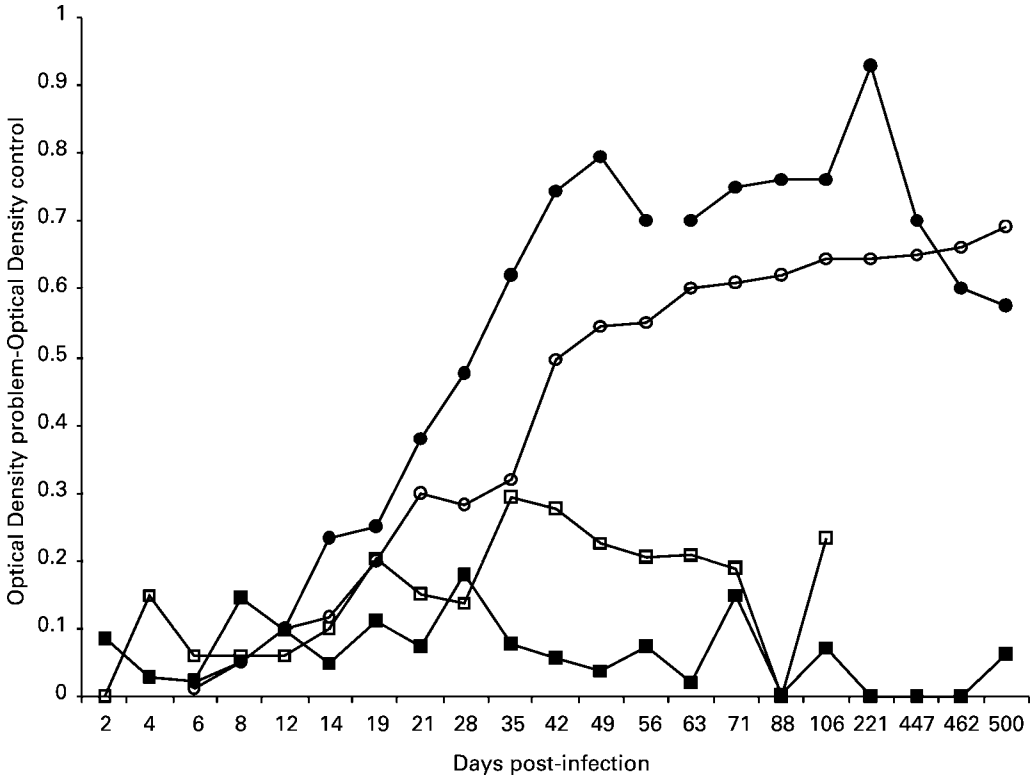

Fig. 1. Dynamics of Ig(G+M+A) responses by ELISA in BALB/c (closed symbols) and C57BL/10 (open symbols) mice inoculated with a single dose of *Anisakis simplex* living third stage larva (■ and □) or *Toxocara canis* embryonated eggs (● and ○) in a single dose, against *T. canis* excretory–secretory antigen.

cross-reactivity between common antigens from related nematodes.

Finally, in the murine model, humoral responses obtained against homologous ES products were higher in the group inoculated with *T. canis* eggs (fig. 1) than those obtained with *A. simplex* (Perteguer & Cuéllar, 1998). Therefore, *T. canis* ES products show a higher reactivity, not only with heterologous but also with homologous sera. This high reactivity shown by *T. canis* ES products could explain our previous results observed in human clinical trials (Perteguer *et al.*, 2003) in which sera from patients diagnosed with sensitivity to *Anisakis* recognized *T. canis* ES products with greater intensity than homologous ones.

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