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Toxocara canis infections in a pig model: immunological, haematological and blood biochemistry responses

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Abstract

The immunological, haematological and enzymatic responses to the inoculation in pigs of 100,000 embryonated eggs of *Toxocara canis* were studied. Fifteen females were inoculated and three remained as controls. Haematological values were analysed from day 7 p.i. until day 126 p.i. In the inoculated group, white blood cells were raised on day 14 p.i. and eosinophil values on days 7, 14, 21, 35 and 49 p.i. showing significant differences compared with controls ($P < 0.05$). Absolute eosinophil counts (per ml) presented two rises, the first on days 7, 14 and 21 p.i. and the second on days 35 and 49 p.i. Blood biochemistry was maintained within normal values. Serological examination by ELISA to determine antibody levels against *Toxocara canis* L2/L3 excretory–secretory (ES) antigens showed values higher than the positive cut-off (1:32) from day 7 p.i. and until the end of the study on day 126 p.i., presenting two peaks: one on day 28 p.i. and the second covering days 49 to 56 p.i. Western blots of sera of inoculated animals presented, from day 7 p.i., two polypeptide bands of 55 and 70 kDa MW and, from day 56 p.i., an additional band of 120 kDa MW, all of which persisted until the end of the study. Immunological responses were sustained over time. No direct correlation was observed between the rise in eosinophils and antibody titres. To validate the conclusions, more studies are required on the polypeptide bands.

Introduction

Toxocara canis is a nematode whose biological cycle includes its definitive canine host, the environment and paratenic hosts, which include man, rats, rabbits, birds and pigs. Diagnosis in paratenic hosts, where the cycle is incomplete, is carried out using serological tests, biopsies and necropsies (Glickman, 1993).

Various animal models, inoculated with different doses of *Toxocara canis* embryonated eggs, have been used to evaluate haematological and/or serological parameters (Ronéus, 1966; Bisseru, 1969; Zapart & Przyjalkowski, 1976; Glickman & Summers, 1983; Basualdo Farjat *et al.*, 1995; Helwigh *et al.*, 1999; Muñoz *et al.*, 1999a,b; Sommerfelt *et al.*, 2001; Taira *et al.*, 2003). An appropriate animal model must imitate mainly the human host, the parasite's responses to the human host or the way both host and parasite interact (Boes & Helwigh, 2000). The pig is an appropriate model for studying the man–*Toxocara canis* relationship (Pond & Houpt, 1979; Helwigh *et al.*, 1999; Swindle & Smith, 2000; Sommerfelt *et al.*, 2001; Taira *et al.*, 2003).

The objective of this study was to evaluate the immunological, haematological and biochemical

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response of pigs to an experimental inoculation with a high dose of *Toxocara canis* embryonated eggs.

Materials and methods

Toxocara canis *eggs*

Toxocara canis eggs were obtained and embryonated according to that described by Sommerfelt *et al.* (2001). Egg infectivity was verified through the experimental inoculation of mice and the recuperation of larvae in the tissues.

Pigs

Eighteen 42-day-old female pigs of Yorkshire breed, weighing between 17 and 19 kg, were used. They were housed in covered pens in a barn with a concrete floor which was previously washed with concentrated sodium hypochlorite and afterwards sterilized by application of heat. Pigs were fed with a commercial growing ration. Fifteen of 18 pigs were chosen at random to form four sub-groups of infected animals ($n = 15$; inoculated group) while the remaining three were the uninfected control group ($n = 3$).

Pigs were each inoculated with 100,000 infective *T. canis* eggs, using a stomach tube under 0.17 ml of 2% xylacine and 1 ml per 7 kg of ketamine. The stomach tube was washed through with a further 40 ml of water to ensure that all the eggs were deposited in the stomach. Three pigs were not infected and served as controls. Animals were treated according to international ethical standards of animal management (Committee members of the International Society on Toxinology, 1986) and to standards of the Ethical Committee of the School of Veterinary Sciences of the Buenos Aires University, Argentina.

Blood sample collection

On day 0 prior to inoculation and once every seven days until day 126 p.i., 10 ml of blood was extracted by venipuncture from each pig. Of this, 1 ml was placed into tubes containing an anti-coagulant for haematological analysis, and the remaining 9 ml in tubes without additives to obtain sera for enzymatic and immunological studies.

Haematological and enzymatic analysis

Samples were processed immediately. The following studies were carried out: haematocrit (%); haemoglobin concentration (g/dl^{-1}); white blood cells count and ratio (%); and absolute count of eosinophils. Blood biochemistry values were measured by use of an automated spectrophotometer Metrolab 2100 (Metrolab) and included urea (mg/dl^{-1}), creatine (mg/dl^{-1}), total proteins (g/dl^{-1}), albumin (g/dl^{-1}), globulin (g/dl^{-1}), albumin:globulin ratio, alanine aminotransferase (UI/l^{-1}), aspartate aminotransferase (UI/l^{-1}), gamma glutamyltransferase (UI/l^{-1}), creatinine kinase (UI/l^{-1}) and alkaline phosphatase (UI/l^{-1}). Values described by Jain (1986) were taken as normal for haematology and those described by Kaneko (1989) as normal for blood biochemistry.

Antigen preparation

The antigen for *T. canis* was prepared according to the technique described by De Savigny (1975). The antigen for diagnosis of *Ascaris suum* was made by obtaining eggs of the parasite from adult females and then following the procedure described by De Savigny (1975).

Porcine serum was adsorbed with equal parts of ES antigen of *Ascaris suum* to eliminate proteins common to the ES antigen of *T. canis* and to avoid possible cross reactions. Adsorption was carried out for 1 h at 37°C and then centrifuged at 3000 rpm for 10 min.

ELISA

The ELISA technique was developed according to the method described by Coltorti *et al.* (1990), with the corresponding modifications for the diagnosis of toxocariasis (Sommerfelt *et al.*, 2001). The positive cut-off value was calculated using sera collected at day 0 from the control group. Results were expressed as dilutions, with titres equal to or higher than 1:32 regarded as positive.

Western blot

Electrophoresis separation and the transfer of antigen to a nitrocellulose membrane were carried out according to Magnaval *et al.* (1991). The TES Ag was diluted in Tris pH 8.6 dissociating buffer (Tris 0.3 g, SDS 0.1 g, glycine 1.25 g supplemented with distilled water to a volume of 100 ml). Electrophoresis was carried out in a Miniprotean II BIORAD, the resolving gel containing 10% polyacrylamide. The gel was loaded with 7.5 μg TES Ag per lane, with the current set at 15 mA. Relative molecular weights were calculated using pre-stained protein molecular weight standards (Bio Rad). The transfer was performed in a transblot cell (Bio Rad) for 1 h to 150v. The nitrocellulose membrane was blocked overnight with 5% skimmed milk in PBST at 4°C. After three washes with PBST, the nitrocellulose filters were incubated with serum diluted 1:100 in PBST plus 1.5% skimmed milk for 1 h at room temperature. The membranes were washed again, then incubated with anti-pig IgG conjugated with horseradish peroxidase (Sigma A 9417, St Louis, Missouri) diluted 1:1500 in PBST plus 1.5% skimmed milk for 1 h at room temperature. For colour development the membranes were washed three times with PBST and immersed in a solution of 3, 3′-diaminobenzyline (DAB). After 5 min the reaction was stopped by washing with distilled water.

Statistical analyses

Data collected were compared with normal values and to those of the control group using an analysis of variance (ANOVA). Levels of significance were set at $P < 0.05$ and were corrected by a Greenhouse and Geisser test. Antibody titres were expressed in a logarithmic scale. Comparison of eosinophil values was carried out using the Bonferroni test.

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Results

Haematological and enzymatic values

Results of haematocrit (%), haemoglobin (g/dl^{-1}), segmented neutrophils (%), band neutrophils (%), lymphocytes (%) and monocytes (%) were considered normal without any significant difference being observed between control and inoculated groups.

White blood cells only rose above normal values on day 14 p.i. in the inoculated group. Eosinophil values were found to be higher in inoculated pigs on days 7, 14, 21, 35 and 49 p.i., with significant differences being observed with the control group ($P < 0.05$) (fig. 1). Absolute eosinophil count (per ml) showed two rises in the inoculated group, the first on days 7, 14 and 21 p.i. and the second on days 35 and 49 p.i. (fig. 2), with differences being significant ($P < 0.05$) with respect to the control group.

Blood biochemistry results were within normal values for the species and presented neither significant differences nor definite tendencies in any case between control and inoculated groups.

ELISA detection of IgG response in pigs

An increase in the level of antibodies against the ES antigen for *Toxocara canis* L2/L3 was observed from day 7 p.i., remaining above the cut-off value until the end of the experiment (day 126 p.i.). An increase in the level of antibodies was seen on day 28 p.i. with a maximum peak on days 49 and 56 p.i. After this, values maintained constant until day 126 p.i. (fig. 3). Values for the control group were always below the positive cut-off value.

Western blot

Results for the Western blot carried out on all serum samples studied are presented in fig. 4. On day 0, prior to inoculation, no polypeptide bands were observed in sera of inoculated pigs (row 1), whereas sera from controls did not present any bands throughout the study (row 3). On day 7 p.i., all sera from the inoculated group presented two polypeptide bands of 55 and 70 kDa MW (rows 4 to 17) and these remained throughout the study. From day 56 p.i. another band of 120 kDa MW was added (row 10 to 17) which also remained throughout the study.

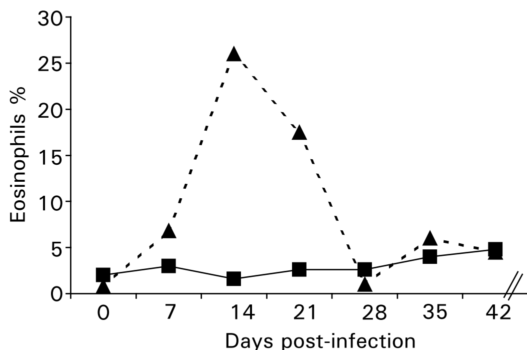

Fig. 1. Values of eosinophils (%) in pigs infected with 100,000 embryonated *Toxocara canis* eggs and in uninfected pigs (■, uninfected pigs; ▲, infected pigs).

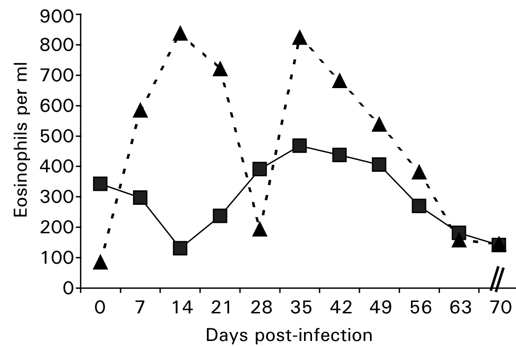

Fig. 2. Values of absolute eosinophil count in pigs infected with 100,000 embryonated *Toxocara canis* eggs and in uninfected pigs (■, uninfected pigs; ▲, infected pigs).

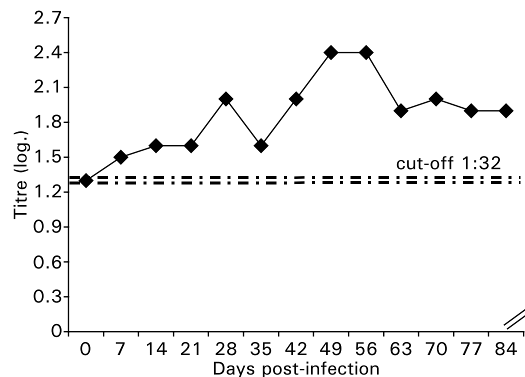

Fig. 3. Development of specific *Toxocara canis* L2/L3 ES antigen in pigs infected with 100,000 *T. canis* eggs. Values of the uninfected group were lower than the positive cut-off 1:32.

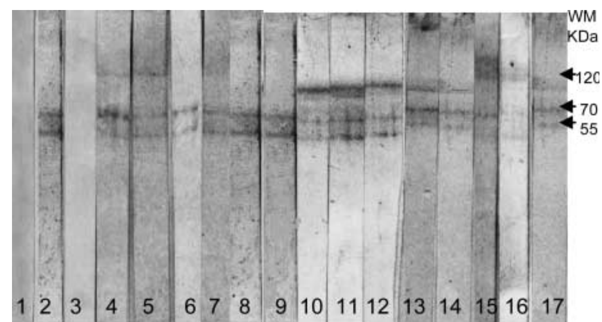

Fig. 4. Western blot analyses of the sera of pigs. Row 1, control negative group; row 2, control positive; row 3, group before becoming infected with *Toxocara canis*; rows 4–9, days 7 to 49 p.i.; rows 10–17, days 56 to 126 p.i.

Discussion

Relative eosinophil values (%) and absolute eosinophil counts (per ml) appeared to be sensitive in the diagnosis of the infection and its evolution. The present observations coincide with those of Ronéus (1966) who observed an increase in eosinophils in pigs from day

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6 p.i. rising to a maximum level on day 15 p.i. Helwigh *et al.* (1999) inoculated pigs and observed increased eosinophil levels during the first week, decreasing as from day 28 p.i. Sommerfelt *et al.* (2001), when inoculating pigs with low doses of *T. canis,* obtained high levels of eosinophils between the second and seventh week p.i. in both groups and a significantly higher value of absolute eosinophils count in the group inoculated with 2000 eggs between weeks 2 and 4 p.i. Taira *et al.* (2003), inoculated groups of pigs with different doses, i.e. a single dose of 50,000 *T. canis* eggs, a dose of 50,000 *T. canis* eggs followed by a challenge infection with 10,000 eggs, or a challenge infection of 10,000 *T. canis* eggs. They observed a significant eosinophil increase on day 14 p.i. in the first group, while in the other groups, the eosinophil count was carried out from day 35 p.i., and responses were quite variable, with no statistical differences being observed. Bisseru (1969), when studying the haematological response in mice inoculated with *T. canis*, observed an increase in eosinophils 7 days p.i. and normal values on day 21 p.i. Different results were obtained when inoculating a *Macaca irus* monkey with 2500 eggs. In this case, eosinophils remained high for four months, with highest values being reached by the second week p.i. (Bisseru, 1969). Zapart & Przyjalkowski (1976) inoculated mice with varying doses and eosinophil values were increased, with a maximum registered on day 6 p.i. Glickman & Summers (1983) administered 45,000 *T. canis* eggs to monkeys (*Macaca fascicularis*) with single and repeated doses and observed that eosinophil values increased on day 14 p.i., peaked on day 29 p.i., and maintained high values during the seven month period of study. Basualdo Farjat *et al.* (1995) observed an increase in eosinophils in mice 8 days p.i. and then again on 14 and 21 days p.i. Results coincide in that an increase in eosinophil values is produced during the first phase of infection, the acute stage, the larvae migration stage, but this trend is not present in chronic stages. This is different to that observed in monkeys (Bisseru, 1969; Glickman & Summers, 1983) and in humans (Smith & Beaver, 1953) where eosinophilia is present in chronic infections. We agree with Taira *et al.* (2003) in that eosinophilia is an infection response that may be different between humans and pigs.

Enzymatic values remained within a normal range and similar to those reported by Ronéus (1966), Sommerfelt *et al.* (2001) and Taira *et al.* (2003). In the case of Glickman & Summers (1983), a rise in the value of alanine aminotransferase (ALT) was seen in monkeys 4 days p.i. which lasted for six months. In this case, experimental conditions differed in the dose administered and the species used. An immunological response was early, 7 days p.i., and was maintained throughout the study (4 months) and these results are similar to those of other authors. Glickman & Summers (1983) detected specific antibodies against *T. canis* with ELISA (cut-off 1:32), in three monkeys on day 4 p.i., in six monkeys on day 7 and in the remaining seven monkeys on day 29 p.i., with high titres persisting over a seven month period. Bowman *et al.* (1987), when inoculating mice, detected antibodies against *T. canis* with ELISA from the first week and up to 26 weeks p.i.. Helwigh *et al.* (1999) detected antibodies in pigs between days 7 and 14 p.i., which remained high until day 28 p.i. Santillán *et al.* (2000), in young dogs naturally infected with *T. canis*, detected with ELISA titres of 1:32 in ten animals and of 1:64 in another two. Sommerfelt *et al.* (2001), when inoculating pigs, observed high titres as from day 7 p.i. and up to day 56 p.i. Taira *et al.* (2003) observed an increase in antibody titres from day 14 p.i. in pigs which were maintained over time (day 49). Fenoy *et al.* (1992) reported that antibody titres remain high in man for a period of five years. These results are evidence of a persistence of antibody titres in the different mammalian species used, and similar to those observed in man.

Using Western blot, from day 7 of the infection polypeptide bands of 55 and 70 kDa MW were observed, with the addition of another band of 120 kDa MW on day 56 p.i. which remained throughout the study. Magnaval *et al.* (1991) proposed bands of 24, 28, 30 and 35 kDa for diagnosis in man, while Courtade *et al.* (1995) postulated that the presence of high molecular weight bands could be due to cross reactions. Muñoz *et al.* (1999 a,b) described bands of 28, 32, 48 and 66 kDa in rabbits and bands of 16, 20, 23, 24, 25, 32, 38, 47, 66, 74, 86, 120 and 200 kDa in naturally infected pets. Santillán *et al.* (2000) observed bands of 30, 50 and 70 kDa in naturally infected puppies and bands of 30, 32, 55, 70 and 120 kDa in one adult canine. Sommerfelt *et al.* (2001), in pigs infected with 1000 and 2000 *T. canis* eggs, observed bands of 55 and 70 kDa. Possibly antibodies against the other glycoproteins are formed later, as glycoproteins of 32 kDa are anchor proteins of the membrane of *Toxocara* while those of 120 kDa are secreted by the accessory glands and those of 70 and 55 kDa are produced by the secretory pore and cover the surface of the nematode (Maizels *et al.*, 1993). Therefore it is possible that the first polypeptides to stimulate the immune system are those of 70 and 55 kDa because their spatial distribution allows them to be in contact with the host's immune system before those of 32 kDa. Thus, the lower molecular weight bands are not observed during all stages of the disease. Another possibility is that these bands are not visible to the immune system of experimentally infected pigs. Another cause could be the existence of different strains, because in studies by other authors the 32 kDa glycoprotein is positive to hydrocarbon staining for some and not for others (Meghji & Maizels, 1986; Page *et al.*, 1992a). Much variability can be seen in the results from the different species studied as well as when compared to those from man. This reinforces the concept that the immune system of each host recognizes different polypeptide bands.

The present results indicate a sustained immunological response in pigs infected with *T. canis*, which remains during the chronic stage, similar to that observed in man. No direct constant correlation was observed between the increase in eosinophils and antibody titres. Haematological responses in the pig differ from those in man. Polypeptide bands require further studies to validate conclusions. Blood biochemistry responses did not allow measurement of the damage produced by larval migration or later evolution. Enzyme values were not an appropriate indicator for diagnosing the infection. Further research will allow us to determine if the sole presence of high titres is sufficient for the diagnosis and treatment of human toxocariasis.

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