HELMINTHOLOGIA, 41, 1: 3 – 7, 2004

Antibody-mediated response in dogs experimentally infected with *Toxocara canis*: effect of procodazole

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Summary

Procodazole (1H-benzimidazole-2-propanoic acid) is an immunomodulator that increases disease resistance. A study to evaluate the effect of procodazole on the antibody-mediated response was carried out in puppies infected with *Toxocara canis* embryonated eggs. The antibody response (antibody activity in association with IgG and with the IgG1 and IgG2 isotypes) against *T. canis* was established by enzyme-linked immunosorbent assay (ELISA) and crude antigen. One group of dogs was previously treated with procodazole (G-E), prior to and after the infection. Another infected group remained without treatment (G-I), and one group of non-treated and non-infected animals was left as control (G-C). Animals of G-E and G-I remained infected throughout 14 weeks, when all infected animals received piperazine. Procodazole reduced significantly the number of worms recovered in G-E and delayed spontaneous worm elimination. Total IgG response was higher in G-E than that of G-I, whereas IgG1 and IgG2 levels were lower than that of G-I. A dichotomous IgG1/IgG2 response was observed in the two infected groups: first, secretion of IgG2 was stimulated, but when infection progressed this response decreased and IgG1 levels increased.

Key words: *Toxocara canis*; puppies; IgG subclasses; experimental infection; procodazole; ELISA

Introduction

Toxocara canis is the common roundworm which lives in the intestine of almost all new-born puppies and kittens, because older dogs and cats develop a strong immunity to the worms. When a dog has been infected previously and ingests infective eggs, most of larvae that hatch from the eggs do not develop into adults. Rather, they remain in the dog's tissues as second stage somatic larvae. If these second stage somatic larvae are in a female and it gets pregnant, transplacental infection of the foetus will occur (Barriga, 1991).

The relative contribution of mechanical and immunological factors as barriers to infection by *T. canis* is uncertain. There is little information available on the immune response of dogs to infection with *T. canis*. During toxocarosis, spontaneous elimination of worms takes place (Lloyd, 1986; Paz *et al*., 1999). No clear association has been demonstrated between immunological responses and the migratory behaviour of the larvae of this nematode or protection against reinfection with *T. canis*. The question whether antibody-mediated or cell-mediated responses to parasites are predominantly protective or harmful to the host remains unresolved. Deplazes *et al.* (1995) proved that, during its development, *T. canis* releases substances that stimulate the canine B lymphocyte response to the production of various subclasses of IgG. They also indicated the possible existence of a dichotomous immune response in canine toxocariosis. In mice helminthosis, Mosmann and Coffman (1989) proved that IgG1 is associated to Th2 cells response, and IgG2 to Th1 cells response.

Procodazole enhances the phagocytic activity in the liver and the spleen (Fernández *et al*., 1976). In a previous work (Paz *et al.*, 1999) we observed that the administration of procodazole had no noticeable influence on leukocyte production, because the values of neutrophils, lymphocytes and eosinophils were very similar in treated-infected puppies and in untreated-infected ones. It has been proved that the traditional effectors of helminth biology (eosinophils, mast cells) are not required for host defence (Locksley, 1994).

The main objective of the present work was to analyse the effect of procodazole on the course of canine toxocariosis, especially on the antibody-mediated response. For this pur

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pose, one group of puppies was daily treated with procodazole, during thirty days, and infected with *T. canis* embryonated eggs. Another untreated group was infected. One group of non-treated and non-infected animals was left as control. The antibody activity was measured in association with IgG and with the IgG1 and IgG2 subclasses, as well as their relationship with the parasitic burden and egg-output.

Materials and Methods

Dogs and infections

Three-month-old Beagles born and raised under parasite-free conditions at the Faculty of Veterinary of Lugo were orally infected with 3000 embryonated *T. canis* eggs. Infective dose was administered in 2 ml saline solution. Faecal examination four weeks prior to infection showed that these animals did not pass eggs by faeces. Six animals (G-E) were orally treated on each of 28 consecutive days with 50 mg procodazole (Estimulocel®, Laboratorios Alonga, Madrid, Spain). These puppies were infected on the 15th day of treatment. Five dogs in Group I (G-I) were untreated with procodazole but infected with *T. canis*, while four dogs of Group C (G-C) were uninfected, untreated controls. All animals were housed individually.

Faecal samples were taken weekly, from 4 weeks before infection, to estimate the number of *T. canis* eggs per gram of faeces by flotation technique (Paz *et al*., 1999). Each dog was bled weekly to evaluate the antibody-mediated response, from 4 weeks before infection to the 14th week after infection. Adult *T. canis* worms were recovered from the faeces of infected dogs following treatment with piperazine anthelmintic (Cyanamid, Madrid, Spain) (110 mg kg p.v.$^{-1}$) 14 weeks after infection. Faecal examinations were carried out to ensure that all *Toxocara* worms had been expulsed by the action of the anthelmintic and the puppies remained helminth-free.

Somatic antigens (SA)

T. canis adult worms, collected from the intestine of pups, were washed in 0.1M phosphate buffered saline (PBS, pH 7.4) and fragmented in a Polytron tissue homogeneizer (Polytron®, Kinematica, Luzern, Switzerland). After five 15 second ultrasonic bursts, the antigen was delipidized with *n*-hexane. The homogenate was centrifuged at 10000 x *g* for 30 min and the supernatant collected and lyophilised (Paz *et al.*, 1999).

Enzyme linked immunosorbent assay (ELISA)

Antibody activity was measured in association with IgG and the IgG1 and the IgG2 isotypes against SA from *T. canis* adult worms using an indirect-ELISA (Paz *et al.*, 1999). All ELISAs were optimised with regard to antigen concentrations, sera and conjugate dilutions. Positive control sera of dogs with parasitologically proven infection and negative control sera of helminth-free dogs were included in all tests.

Wells of microtiter-plates (Nunc®, Roskilde, Denmark) were coated with 100 µl SA (1.5 µg protein ml^{-1}), sera (in duplicate) were added at 1/60, and the horseradish-peroxidase-conjugated (HRP-conjugated) sheep antidog IgG, IgG1 or IgG2 (H&L, Bethyl Laboratories Inc., Montgomery, USA) at 1/1000. Absorbance was determined using a spectrophotometer (Titertek Multiskan®, Hämeenlinna, Finland) at 492 nm.

Statistical analysis of the differences between the levels of the IgG subclasses was determined by using the analysis of variance (ANOVA) test. Probability values above 0.05 were considered not significant. All tests were done using the statistical package SPSS, version 10.0.6 (SPSS 2001).

Percentages of reduction

To evaluate the effect of procodazole on the egg elimination and on the parasitic burden, the percentages of reduction 1 (PR 1) and 2 (PR 2) were calculated as follows:

$$PR1 = \frac{\text{Number of eggs in G-I} - \text{Number of eggs in G-E}}{\text{Number of eggs in G-I}} \times 100$$

$$PR2 = \frac{\text{Number of worms in G-I} - \text{Number of worms in G-E}}{\text{Number of eggs in G-I}} \times 100$$

Results

Development of infection

Egg-output following infection with *T. canis* began on the 5th week after infection (w.a.i.) in the dogs of G-I and on the 4th w.a.i. in the G-E (Table 1). All infected animals passed eggs in faeces, showing that infection was successful. These results were confirmed on the 14th w.a.i. because *T. canis* adult worms were recovered from all parasitised dogs.

The numbers of egg-output were higher in G-I than in G-E (Table 1). By means of the percentage of reduction 1 (PR1), we observed that in G-E egg-elimination reduced between 0.6 % (9th w.a.i.) and 65 % (10th w.a.i.). Significant differences were obtained in the egg-excretion between G-I and G-E on weeks 6, 13 and 14 after infection.

Table 2 summarises the number of nematodes of G-I and G-E. The mean number of worms was 23.2 ± 15.35 in G-I and 7 ± 7.15 in G-E. ANOVA showed that these differences were significant (F=5.37, P=0.046). The ratio female/male worms was 1.46 in G-I and 1.47 in G-E. Significant differences were obtained in the number of male worms recovered after the administration of piperazine, and in the total male worms obtained ($P < 0.05$).

Spontaneous worm elimination occurred between the 6th and 10th w.a.i. in G-I, and between the 9th and 13th w.a.i. in G-E. The number of worms spontaneously recovered was higher in G-I (63) than in G-E (17) (Table 2), and also the percentage of worms spontaneously expelled (54.3 % in G-I, 40.5 % in G-E).

The percentage of reduction PR2 was 73 % in the worms spontaneously recovered and 52.8 % in the worms obtain-

Table 1. Egg-excretion of puppies infected with *T canis*

Weeks after infection	Eggs per gram of faeces ($\overline{X}$ ± SD)		% of reduction 1	ANOVA	
	G-I	G-E	(PR 1) (%)	F	p
0					
1					
2					
3					
4		400 ± 50.21			
5	260 ± 160.54	260 ± 212.31	0		
6	143 ± 108.79	290 ± 23.12	-102.8		0.048
7	501 ± 328.51	392 ± 321.24	21.7		
8	2000 ± 218.45	1539 ± 581.56	23.05		
9	1853 ± 941.27	1842 ± 1285.12	0.6		
10	2851 ± 1230.71	1000 ± 729.36	64.9		
11	2708 ± 2092.45	1263 ± 441.12	53.4		
12	3388 ± 2032.85	1253 ± 1087.93	63		
13	2243 ± 147.78	1498 ± 351.26	33.2	11.411	0.28
14	2881 ± 247.37	1369 ± 326.55	52.5	67.757	0.009

Data represent mean values ± SD; G-I: puppies infected with 3000 embryonated *T. canis* eggs; G-E: puppies treated with procodazole and infected with embryonated *T. canis* eggs. PR 1= ((Number of eggs in G-I – Number of eggs in G-E)/ Number of eggs in G-I) x 100

Table 2. Number of *T. canis* worms recovered from the puppies of G-I and G-E. PR 2= ((Number of worms in G-I – Number of worms in G-E)/ Number of worms in G-I) x 100

	G-I Total number ($\overline{X}$ ± SD)	G-E Total number ($\overline{X}$ ± SD)	% of reduction 2 PR 2 (%)	ANOVA F	ANOVA p
Spontaneously recovered worms	63 (12.6 ± 17.96)	17 (2.83 ± 4.66)	73		
Female	43 (14.33 ± 15.04)	11 (3.66 ± 4.72)	74.4		
Male	20 (6.66 ± 4.93)		70		
Piperazine recovered worms	53 (10.6 ± 7.33)	25 (4.16 ± 3.48)	52.8		
Female	26 (5.2 ± 4.54)	14 (2.80 ± 2.04)	46.1		
Male	27 (6.75 ± 1.70)	11 (2.75 ± 2.21)	59.2	8.17	0.029
Total worms recovered	116 (23.20 ± 15.35)	42 (7 ± 7.15)	63.8	5.37	0.046
Female	69 (14 ± 11.25)	25 (5.2 ± 4.55)	63.8		
Male	47 (9.2 ± 4.15)	17 (3.40 ± 3.36)	63.8		

ed after the administration of piperazine. The total percentage of reduction was 63.8 %.

Serum antibody responses in the puppies infected with T. canis and in the controls

Results shown in Fig. 1 indicate that specific IgG responses were induced in all orally infected dogs, with no specific response in control ones. In G-I, IgG antibodies increased above the control group as soon as 1 week after infection. The absorbance values gradually rose to the 4th w.a.i. and the highest ones were reached at the end of the study (14th w.a.i.).

The IgG1 titres rose in G-I as soon as the 2nd w.a.i., reaching higher values than in G-C (Fig. 2). After increasing,

IgG1 peaked on the 10 – 11th w.a.i., and decreased to the 14th w.a.i.

As Figure 3 represents, IgG2 antibodies increased in G-I from the 1st w.a.i., peaked on the 4 – 5 w.a.i., and decreased to the end of the study.

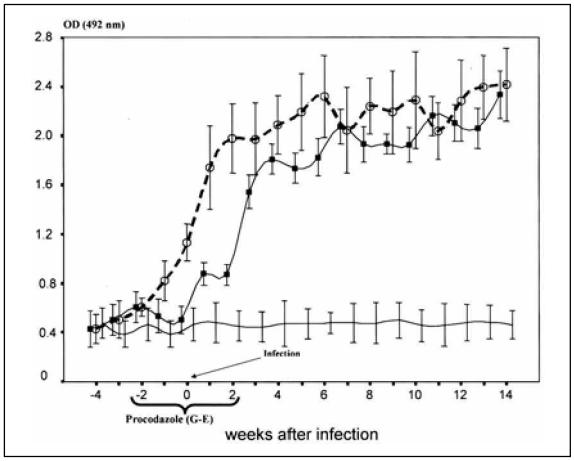

Fig. 1. Serum IgG antibody response to *T. canis* crude antigen
Results are means ± SD; ¢ – dogs infected with embryonated eggs of *T. canis* (G-I); V – dogs treated with procodazole two weeks before and two weeks after infection with embryonated eggs of *T. canis* (G-E); · – uninfected dogs (G-C)

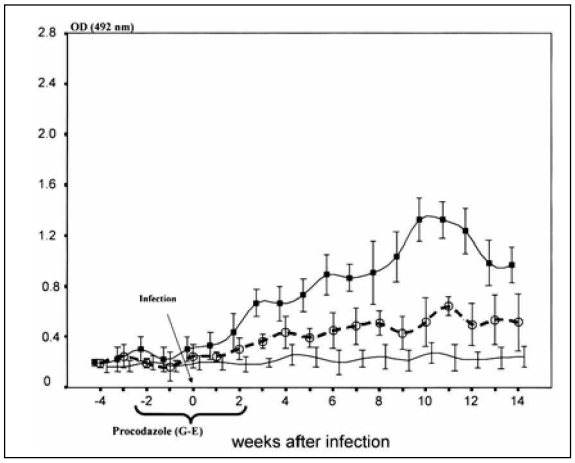

Fig. 2. Serum IgG1 antibody response to *T. canis* crude antigen
Results are means ± SD; ¢ – dogs infected with embryonated eggs of *T. canis* (G-I); V – dogs treated with procodazole two weeks before and two weeks after infection with embryonated eggs of *T. canis* (G-E); · – uninfected dogs (G-C)

Serum antibody responses in the puppies treated with procodazole and infected with T. canis and in the controls

In the puppies of G-E, the values of IgG increased up from one week prior to the infection (Fig. 1), and reached the highest values within the 13th – 14th w.a.i. The differences in the IgG values between G-E and G-I were significant ($F=14$, $P < 0.05$).

Figure 2 shows that IgG1 antibodies showed a similar curve in G-E and in G-I. From the 3rd w.a.i., a marked increment was observed, peaking on the 11th w.a.i. The optical densities of G-E were significantly lower than those of G-I ($F=59.94$, $P < 0.05$).

The IgG2 response rose significantly to the 2nd w.a.i., and maintained a constant value to the end of the study (Fig. 3). The IgG2 values were also significantly lower than those of G-I ($F=64.69$, $P < 0.05$).

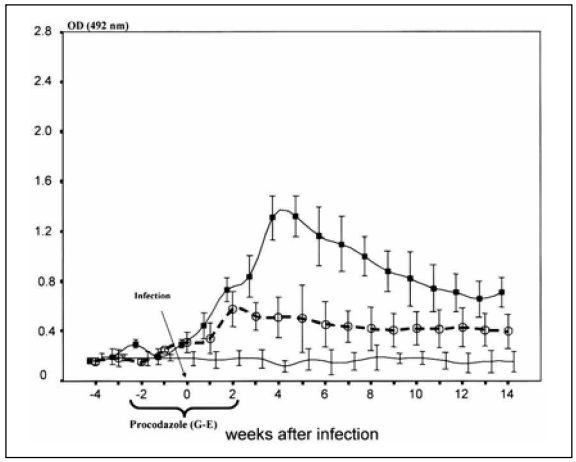

Fig. 3. Serum IgG2 antibody response to *T. canis* crude antigen
Results are means ± SD; ¢ – dogs infected with embryonated eggs of *T. canis* (G-I); V – dogs treated with procodazole two weeks before and two weeks after infection with embryonated eggs of *T. canis* (G-E); · – uninfected dogs (G-C)

Discussion

Procodazole is a widely used immunomodulator in the current clinics, but its activity on the humoral immunity response has not been evaluated. In the current work, one group of puppies was treated with procodazole prior to the infection with *T. canis*-embryonated eggs. The activity of IgG was higher than that of the untreated and infected puppies, but both serum IgG1 and IgG2 responses were lower. The number of worms recovered after the administration of the drug was significantly reduced. Following ingestion by dogs, the *T. canis* infective eggs hatch, larvae penetrate the gut wall and migrate through the liver in the puppies (24-48 hours after infection). One possible explanation to our results could be that the administration of procodazole reduced the number of worms in the first days of infection, which could imply a decrease in the antigenic stimulus responsible for the antibody synthesis, and lower values of IgG1 and IgG2 could be produced. Fernández *et al.* (1976) proved that procodazole stimulates the phagocyte activity and increases the number of mononuclear cells at the liver and the spleen.

One interesting finding was the observation that the spon-

taneous elimination of worms coincided with a decrease in the IgG2 levels, especially in the untreated-infected puppies. This suggests that IgG2 antibodies might be involved in this phenomenon, or that this subclass is stimulated by the infective stages of *T. canis*, when larvae migrate through intestinal wall to the liver and lungs. Once *Toxocara* larvae become L_3 and come back to the intestine, where they develop to L_4 and adult stages, this stimulus might end.

In the current work, a selective downregulation of IgG1 and IgG2 responses in experimental canine toxocarosis was observed, since production of the two subclasses occurred at different times following infection, with IgG2 decreasing as IgG1 rose. Initially, IgG2 was produced in response to the nematode infection, and when infection progressed, nematodes reached the intestine, attained sexual maturity and passed eggs by faeces. The serum levels of IgG2 dropped at the same time as serum IGG1 increased. These results agree with that from Deplazes *et al*. (1995), who demonstrated that IgG1 and IgG2 antibodies in dogs are antagonistically regulated.

The administration of procodazole reduced the parasitic burden, but IgG1 and IgG2 did not increased after administrating the drug. Further studies are needed to get more information on these aspects, and also about the mechanisms responsible for the spontaneous worm elimination.

RECEIVED DECEMBER 5, 2002

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ACCEPTED JULY 1, 2003