Journal of Helminthology (2001) **75,** 175–181 DOI: 10.1079/JOH200178

The influence of inoculum size and time post-infection on the number and position of *Toxocara canis* larvae recovered from the brains of outbred CD1 mice

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Abstract

Outbred CD1 mice were administered doses of 1000 and 3000 *Toxocara canis* eggs and postmortem took place on days 7, 42 and 120 post-infection. Mice were killed by cervical dislocation and brains were sagitally bisected and fixed in 10% neutral buffered formalin prior to histological preparation and examination. The number of *T. canis* larvae were counted per brain and per section and the number of larvae cited for the first time per section were also recorded. These observations were compared by dose administered and by day of postmortem. The total number of larvae per brain and per section was higher for the 3000 dose compared to the 1000 dose. A different pattern emerged for the number of larvae observed in the brain over the three postmortem days depending upon the dose received. For the 1000 dose larval numbers increase from day 7 to day 120 whereas for the 3000 dose the opposite trend occurs. Larvae were assigned to one of five regions in the brain – the telencephalon, diencephalon, cerebellum, medulla, pons and brain stem and the olfactory bulb. Larvae did not show a random distribution in the brain. The majority of larvae were recorded from the telencephalon and the cerebellum. The percentage of sections with larvae in them is higher for the 3000 dose compared to the 1000 dose for all regions of the brain. For the majority of regions, the percentage of sections with larvae in them increases between day 7 and 42 and then decreases by day 120 and this is most pronounced for the cerebellum. For the telencephalon and diencephalon only, more larvae were detected on the right hand side of the brain compared to the left hand side. Statistical analysis revealed that dose and brain region are significant factors which influence the number of larvae observed in histological sections of the brain but day post-infection is not.

Introduction

Toxocara canis is a nematode which infects canines worldwide and, as a consequence of the widespread environmental dissemination of its ova in host faeces, other abnormal hosts including mice and humans are exposed to the parasite (Glickman & Schantz, 1981). In the abnormal or paratenic host, the immature second-stage larvae of the parasite undergoes a somatic migration through the organs of the body but fails to reach maturity as adult worms in the intestine. In humans, attention has focused upon several recognized clinical entities associated with toxocariasis, i.e. visceral larva migrans (Beaver *et al.*, 1952), ocular larva migrans (Shields, 1984) and covert toxocariasis (Taylor *et al.*,

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1988). A phenomenon of potential public health significance in humans and of ecological significance in mice is the evidence that larvae exhibit neurotrophic behaviour, which results in a greater concentration of parasites in the brain as the infection progresses (Sprent, 1955; Dunsmore *et al.*, 1983).

The mouse model has been widely used in the study of toxocariasis due to the similarities seen between the progression of *T. canis* infection in the mouse and in the human (Smith, 1991). Furthermore, small rodents captured from the wild have been shown to harbour *Toxocara* larvae in their tissues and have been hypothesized to contribute to parasite transmission if consumed by an appropriate definitive host (Dubinsky *et al.*, 1995). Recently, the laboratory mouse model has received further attention in that it can be used to test the hypothesis that parasite-altered host behaviour may contribute to increased predation of infected paratenic hosts and hence enhanced transmission of the parasite (Cox & Holland, 1998, 2001a,b). Skerrett & Holland (1997) demonstrated that significant variation in the numbers of larvae recovered from individual mice brains might influence observed changes in behaviour. Cox & Holland (1998, 2001a,b) described how a broad range of behaviours in mice were influenced by the larval burden in the individual mouse brain and concluded that in studies involving parasites with a predilection for the CNS, the data may be best presented in the light of the number of parasites in the brain at the time of testing in addition to the dose administered. One question that arises from this work is the relationship between observed behavioural alterations in *T. canis*-infected mice, the number of larvae recovered from the brain and the site of the larvae within the brain.

Several studies, based upon a single dose of *T. canis* eggs, reported larvae in a non-random distribution within the brain (Burren, 1971; Dolinsky *et al.*, 1981; Summers *et al.*, 1983). Burren (1971) divided the brain into cerebral hemisperes, brain stem and cerebellum and counted larvae by means of brain squashes. Dolinsky *et al.* (1981) used brain squashes to count larvae and histological sections to chart pathology in the presence of larvae. Summers *et al.* (1983) used brain squashes to count larval numbers and histology to detail pathology relative to brain location. To our knowledge, no study has used histology to simultaneously examine both the number and position of larvae in the mouse brain. Furthermore, we compare data from mice which received two doses of infective eggs and underwent postmortem on three days post-infection, the last day of which represented a longer time period compared to most experiments.

Materials and methods

Infection of mice

Groups of six- to eight-week-old CD1-ICR outbred male mice (Harlan Laboratories, UK) were infected orally by stomach intubation with doses of 100, 1000, 3000 and trickle (100–125 eggs on a weekly basis to make up a total dose of 1000) *T. canis* eggs suspended in 0.2 ml of 0.85% physiological saline. The control groups were sham-inoculated with distilled water. Mice were subsequently killed by cervical dislocation on days 1, 2, 3, 7, 14, 21, 28, 35, 42 and 119 ($n = 200$). Of the five mice killed within each day and dose group, the tissues of three mice were processed for larval migration while the tissues of two mice were fixed for histology. This paper reports the histological findings from the brains of 12 mice, two mice in each of two of the dose groups (1000 and 3000 eggs) which were sacrificed on days 7, 42 and 120 post-infection.

Brain histology and light microscopy

All brains were sagitally bisected, fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin wax, serially sectioned at 6 μm and every alternate slide (which contained three to four brain sections) stained with either haematoxylin and eosin or Cresyl Fast Violet. A minimum of two sections (of each brain hemisphere) were examined every 30 μm for the presence and distribution of larvae. To ensure larvae that appeared in serial sections were not counted more than once, the position of larvae were marked on diagrams of the brain taken from the atlas of the mouse brain (Sidman *et al.*, 1971). Larvae were assigned to one of five regions in the brain – the telencephalon, diencephalon, mesencephalon, cerebellum, medulla, pons and brain stem or olfactory bulb (Sidman *et al.*, 1971) (see fig. 1).

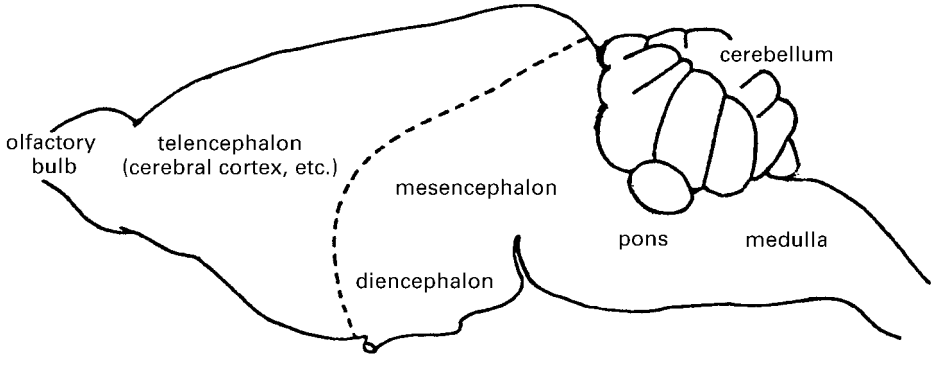

Fig. 1. Lateral view of mouse brain with the regions of the brain used in the analysis marked (adapted from Sidman *et al.*, 1971).

Table 1. The mean number of *Toxocara canis* larvae (±SD) observed per brain in the two dose groups over the three days post-infection.

Dose group	Day post-infection	No. of brains examined	Mean no. of larvae per brain (±SD)	Number of larvae expressed as a % of original dose
1000	7	2	13 (2.8)	1.3
1000	42	2	16 (3.5)	1.6
1000	120	2	27 (3.5)	2.7
3000	7	2	50 (19.1)	1.7
3000	42	2	67 (7.1)	2.2
3000	120	2	39 (0.7)	1.3

Statistical methods

All statistical tests were carried out at the 95% confidence limit. The numbers of larvae recovered from the brains of mice were found to be not normally distributed so the data was log transformed prior to analysis. Two-way analysis of variance (ANOVA) was used to explore the relationship between the number of larvae recovered from the mice brains and the dose of ova administered, the day of postmortem and the interaction between these two factors (dose × day). Chi-square analysis was initially used to explore the relationship between the frequency of larvae in particular regions of the brain and the dose administered, day of postmortem and whether larvae were recorded on the right or left hand side of the brain. Three-way analysis of variance was then used to assess the relationship between the number of larvae recovered from the brain and their location within the brain, the dose of ova administered, the day of postmortem and the interactions between these pairs of factors and all three of them (position × dose × day).

Results

Total number of larvae found in the brain

Larvae were cited in the brain on all post-infection days examined (table 1). The total number of larvae per brain was higher for the 3000 dose compared to the 1000 dose. A different pattern emerged for the total number of larvae observed in the brain over time depending upon the dose of eggs the mice originally received. In the mice which received the 1000 dose, the number of larvae show a steady increase over the experimental period peaking in an average of 27 larvae per brain on day 120. In contrast, for the 3000 dose a slight increase occurs between day 7 and 42 but by day 120 the number of larvae have decreased considerably (i.e. an average of 67 to 39 per brain) (table 1). The number of larvae expressed as a percentage of original dose administered was low regardless of dose size and day of postmortem (table 1).

The number of larvae observed in the brains of infected mice was also examined with respect to the mean number of larvae seen per section and the mean number of larvae cited for the first time ('new larvae') per section (table 2). As was the case for the total number of larvae per brain, the numbers of larvae per section were higher for the 3000 dose compared to the 1000 dose and the pattern over the days followed the same trend (table 2).

Two-way analysis of variance was then used to explore the relationship between the total numbers of larvae observed per brain and dose, day and the dose × day interaction. Dose was found to be highly significant factor, day was not, but the interaction between the two was also significant (dose F ratio = 71, $P \leq 0.0001$; day F ratio = 2, $P \leq 0.1344$: dose × day F ratio = 8, $P \leq 0.0004$).

Distribution of the larvae in the brain

Larvae were observed in the thalamus, capsula interna, capsula externa, fornix, corpus callosum, cerebral cortex,

Table 2. The mean number of *Toxocara canis* larvae (±SD) observed per section in the two dose groups over the three days post-infection.

Dose	Day pi	Number of sections examined per brain	Number of larvae observed per section		Number of new larvae[1] cited per section	
			Mean (±SD)	Max	Mean (±SD)	Max
1000	7	320	0.17 (0.5)	3	0.08 (0.3)	2
1000	42	288	0.30 (0.6)	4	0.10 (0.4)	3
1000	120	400	0.39 (0.7)	4	0.14 (0.4)	3
3000	7	312	0.88 (1.2)	6	0.3 (0.7)	4
3000	42	297	1.4 (1.7)	8	0.4 (0.9)	5
3000	120	370	0.6 (0.8)	5	0.2 (0.5)	3

[1] New larvae: larvae observed for the first time in each section.

Table 3. The mean number of *Toxocara canis* larvae (±SD) observed in the two dose groups over the three days post-infection relative to their position in six regions of the murine brain.

Dose	Day pi	Tel.	Dien.	Mes.	Cer.	Med.	Olf.	Total
1000	7	7 (2.1)	0 (0.0)	0 (0.0)	6 (2.1)	1 (1.4)	0 (0.0)	13 (2.8)
1000	42	12 (6.4)	0 (0.0)	0 (0.0)	4 (2.8)	0 (0.0)	0 (0.0)	16 (3.5)
1000	120	18 (1.4)	5 (2.8)	0 (0.0)	3 (2.8)	1 (0.7)	0 (0.0)	27 (3.5)
3000	7	32 (10.6)	4 (2.8)	0 (0.0)	10 (2.8)	2 (1.4)	2 (1.4)	50 (19.1)
3000	42	43 (7.8)	6 (4.2)	0 (0.0)	15 (5.7)	3 (1.4)	1 (0.7)	67 (7.1)
3000	120	32 (8.4)	2 (2.1)	0 (0.0)	8 (*)	1 (1.4)	0 (0.0)	39 (0.7)

Tel, telencephalon; Dien, diencephalon; Mes, mesencephalon; Cer, cerebellum; Med, medulla, pons & brain stem; Olf, olfactory bulb. *n = 1.

medulla, olfactory bulb, hippocampus and cerebellum. The total number of larvae observed in each of the regions defined in the Materials and methods are shown in table 3. The majority of larvae were recorded from the telencephalon and cerebellum. No larvae were located in the mesencephalon. Within the telencephalon, over 60% of the larvae were located in the corpus callosum. The trend of larval numbers over time varies by brain region, for example in the telencephalon the numbers of larvae increase over time for the 1000 dose but the opposite is true for the cerebellum. At the 3000 dose, larval numbers increase from day 7 to 42 but then remain the same as day 7 for day 120. The decrease is more gradual over the three days for the cerebellum.

The percentage of sections with larvae in them are provided by dose and by day in table 4a,b. The percentage of sections with larvae increases in all regions of the brain as dose increases (table 4a). For the majority of regions, the percentage of sections with larvae in them increases between day 7 and 42 and then decreases by day 120 and this is most pronounced for the cerebellum (table 4b). The percentage of sections with larvae in regions of the brain on the left and right hand side are shown in table 4c. For the telencephalon and diencephalon only, more larvae were detected on the right hand side of the brain compared to the left hand side.

Two analyses were performed upon the positional data. Firstly, Chi-square analysis was used to explore the relationship between the frequency of larvae in particular regions of the brain and the dose administered, day of postmortem and whether larvae were recorded on the right or left hand side of the brain (table 5). This revealed that for all regions of the brain, dose remains very significant as reflected in table 4a with more larvae always being evident in all brain regions for the 3000 dose compared to the 1000 dose. In contrast, day was a statistically significant factor for three of the five regions – the telencephalon, cerebellum and medulla, pons and brain stem (see also table 4b). This is in contrast to the analysis of number of larvae per brain which found day to be an insignificant factor. Brain side was a significant factor for the telencephalon and diencephalon only (see also table 4c).

Secondly, three-way analysis of variance was used to explore the relationship between the total number of larvae observed per brain and the factors dose administered, day of postmortem, brain region and the interaction between each of pair of factors and all three. The results for dose and day are similar to those observed previously for the two-way ANOVA, in addition, as expected the number of larvae observed varied significantly by brain region (table 6). The

Table 4. (a) The percentage of sections with *Toxocara canis* larvae in regions of the brain for each dose group. (b) The percentage of sections with *T. canis* larvae in regions of the brain for days 7, 42 and 120. (c) The percentage of sections with *T. canis* larvae in regions of the brain on the left (LHS) and right hand side (RHS) of the brain.

a

Dose	Tel.	Dien.	Cer.	Med.	Olf.	Total
1000	15.5%	3.0%	6.3%	0.2%	0%	22.3%
No.	156/1008	26/1008	63/1008	2/1008	0/1008	225/1008
3000	39.0%	6.0%	15.6%	3.0%	1.3%	48.5%
No.	378/980	59/980	124/796	28/980	13/980	386/796

b

Day	Tel.	Dien.	Cer.	Med.	Olf.	Total
7	22.2%	3.2%	10.4%	2.2%	1.3%	30.1%
No.	140/632	20/632	66/632	14/632	8/632	91/632
42	31.1%	5.6%	14.2%	2.0%	0.33%	33.4%
No.	190/610	34/610	93/610	12/610	2/610	101/610
120	27.3%	4.2%	5.0%	0.54%	0.40%	33.0%
No.	204/746	31/746	28/562	4/746	3/746	110/746

c

Brain side	Tel.	Dien.	Cer.	Med.	Olf.
LHS	24.7%	3.2%	10.8%	2.0%	0.6%
No.	239/967	31/967	87/875	18/967	6/967
RHS	29.0%	5.3%	10.0%	1.2%	0.7%
No.	295/1021	54/1021	100/929	12/1021	7/1021

No., number of sections wih larvae per total sections examined; Tel, telencephalon; Dien, diencephalon; Mes, mesencephalon; Cer, cerebellum; Med, medulla, pons and brain stem; Olf, olfactory bulb.

Table 5. Results of Chi-square analysis investigating whether the presence or absence of *Toxocara canis* larvae are associated with dose, day post-infection and brain region.

Brain region	Factor	χ^2	*P* value	Fishers exact test	Degrees of freedom
Telencephalon	Dose	134.9	≤0.0001	≤0.0001	1
	Day	12.9	0.0002	0.0016	2
	Brain side	4.4	0.0357	0.0357	1
Diencephalon	Dose	14.4	0.0001	≤0.0001	1
	Day	4.4	0.1084	–	2
	Brain side	5.3	0.0218	0.0380	1
Cerebellum	Dose	41.7	≤0.0001	≤0.0001	1
	Day	33.2	≤0.0001	–	2
	Brain side	0.3	0.5673	0.5891	1
Medulla, pons and brain stem	Dose	23.6	≤0.0001	≤0.0001	1
	Day	7.7	0.0209	–	2
	Brain side	1.6	0.2098	0.2695	1
Olfactory bulb	Dose	13.5	0.0002		1
	Day	5.4	0.0683	–	2
	Brain side	0.03	0.8571	1.0	1

interaction between dose and position was also significant and this mirrors the findings of the Chi-square analysis. The interaction between day and position is not significant and this reflects the weaker relationship by day observed for the Chi-square analysis. The interaction term between all three factors is just significant but is the least so of all the five significant factors (table 6).

Discussion

The number of *Toxocara* second stage larvae were quantified per brain and their position assigned to one of five broad locations in outbred CD1 mice which had received one of two doses of eggs and for whom postmortem had taken place on days 7, 42 or 120. Several previous studies have examined the influence of the inoculum of *Toxocara* eggs upon brain recovery of larvae in mice and it is clear that a range of factors including the strain of mice used, the subsequent immune response, the inoculum given, the duration of infection and the method of larval recovery are likely to influence the observed number of larvae recovered from the murine brain (Kayes & Oaks, 1976; Abo-Shehada & Herbert, 1989; Abo-Shehada *et al.*, 1991; Epe *et al.*, 1994; Cox & Holland, 2001a).

In the present study, the number of larvae per brain and per brain section was significantly, higher for those mice which received the 3000 dose compared to the 1000 dose. Patterns of brain infection differed by day depending upon which dose was examined. For the 1000 dose the number of larvae showed a steady increase or accumulation over time whereas for the 3000 dose the number of larvae decreased. Statistically, day alone was not a significant factor but a significant day × dose interaction was observed. In contrast, Dunsmore *et al.* (1983) demonstrated that the number of larvae in the murine brain of outbred Canberra and inbred C57BL mice, following doses of 1000 and 5000 eggs, increased over time (ranging from day 2 to day 122 depending upon the experiment) and they described this as larval accumulation for both doses. In fact, the accumulation was more pronounced for the 5000 dose compared to the 1000 dose. Burren (1971) reported that Clarke's OS1 mice received a single dose of 1000 eggs and there was evidence of a decline in larval numbers in the brain over time (from day 2 to day 138). Before day 7 larval numbers increased but after that no increase was detected up to day 138.

Table 6. Results of a three-way analysis of variance (ANOVA) investigating the effect of dose, day and position on the number of larvae observed in the brains of *Toxocara canis* infected mice.

Organ	Factor	F ratio	*P* value	Degrees of freedom
Brain	Dose	41.2	≤0.0001	1
	Day	0.4	0.6863	2
	Dose × day	7.0	0.0018	2
	Position	46.4	≤0.0001	11
	Dose × position	2.1	0.0369	11
	Day × position	1.2	0.2580	22
	Dose × day × position	1.7	0.0464	22

Epe *et al.* (1994) utilized four inbred and one outbred strain of mice, selected with special emphasis upon the major histocompatability complex, and these mice received a single dose of 1000 eggs and numbers in the brain (and other sites) were counted for days 4, 8, 12, 17 and 21 respectively. Interestingly, there was no evidence of larval accumulation over time for any of the five strains but there was evidence of differences in larval distribution and pathogenicity between the strains. Bardon *et al.* (1994) noted a reduction in larval numbers, in the carcass of BALB/c mice which had received a 1000 egg dose, one year post-infection compared to day 63 but this reduction was not mirrored in the brain.

Kayes & Oakes (1976) recovered larvae from the brain of HaM/ICR mice which received doses of 200, 600 or 1000 doses of eggs and underwent postmortem on days 7 through to 50 days post-infection. These observations were similar to the present study in that inoculum size was a significant factor in determining the proportional recovery from the brain but the length of infection was also significant, as was the interaction between the two factors. Kayes & Oakes (1976) suggested that fluctuations in larval numbers may reflect the ability of larvae to migrate in and out of the sampled tissues over time or that larvae die and degrade with the larger the inoculum the greater the peak and subsequent decline. Kayes *et al.* (1985) also demonstrated that the magnitude of the anti-toxocaral humoral response is directly proportional to the size of the inoculum given.

In contrast to the observations on the relationship between larval numbers and inoculum size and post-mortem day, much less attention has been paid to the position of *Toxocara* in the mouse brain. In the present study, histology was used to simultaneously examine both the number and position of larvae in the mouse brain and to explore the relationship between position, dose administered and day of postmortem. Three previous papers, namely Burren (1971) Dolinsky *et al.* (1981) and Summers *et al.* (1983) described experiments which utilized a single dose of 1000 ova and examined various aspects of murine cerebral toxocariasis.

Burren (1971) separated the brains of infected mice into cerebral hemispheres, brain stem and cerebellum. Portions of the brain were then squashed between glass plates and examined microscopically. Burren calculated that the average weight of murine cerebral hemispheres was heavier than the cerebellum by a factor of 3.6 yet the former contained fewer larvae per unit weight of tissue. He concluded that in general the number of larvae per 50 mg of tissue was higher in the cerebellum relative to other regions.

In two papers describing various aspects of the same study, Dolinsky *et al.* (1981) and Summers *et al.* (1983) described how brains of Binghamton heterogeneous mice were divided into two – one section of which was divided into portions for squashing and counting of larval numbers, and the other was fixed for histology and examined for the distribution of larvae and the nature of associated lesions. Lesions and parasites were most frequently found in heavily myelinated tracts including the corpus callosum, internal and external capsules, cerebellar peduncles and cerebellar medulla. More sporadically, sitings were recorded in the cerebral and cerebellar cortices, thalamus, hypothalmus, tectum, pons and medulla. In pathological terms, the authors described a striking encephalitis with an affinity for white matter areas including internal and external capsules and cerebellar peduncles. The corpus callosum was the most extensively injured. Summers *et al.* (1983) suggested that the observed non-random distribution might reflect a selective tropism for myelin, a nutritional advantage offered by these lipids for *Toxocara* larvae or simply the pathway of least resistance through the brain.

To conclude, the present paper provides further quantitative evidence of the non-random distribution of *Toxocara* larvae in the murine brain, using histology which is likely to provide a more accurate assessment of both larval numbers and their position. Furthermore, the non-random distribution of larvae is maintained at both a medium and high dose for the telencephalon and cerebellum. There is some evidence that the process of accumulation for the two doses differs over time but this does not achieve statistical significance. We have evidence that for two strains of outbred mice, high doses of eggs produce increased numbers of larvae in the murine brain (Good, 1998; Cox & Holland, 2001a) which are associated with a wide range of behavioural alterations (Cox & Holland, 2001a,b). This present paper provides evidence that at a higher dose, larval position does not differ and hence the manifestation of behavioural alterations are likely to be linked to larval intensity in the brain rather than differential position.

Acknowledgements

This research was funded by a basic research grant from Enterprise Ireland.

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(Accepted 5 March 2001)