

Transfer of immunity to *Nematospiroides dubius*: co-operation between lymphoid cells and antibodies in mediating worm expulsion

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Summary The effect of transferring immune serum (IS) and immune mesenteric lymph node cells (IMLNC) either alone or in combination was studied in NIH mice infected with partially radiation attenuated (5 krad) *N. dubius*. It was demonstrated that immunity to *N. dubius* could be transferred with IS and with IMLNC. However, considerably greater protection was transferred to recipient mice when they received both IS + IMLNC. Animals treated in this way had fewer worms than either of the other groups from as early as day 9 onwards, suggesting that a substantial proportion of the worms in mice given IS + IMLNC was retained in the intestinal tissues. The few surviving worms which completed the tissue phase of their development were then rejected by the fourth week of infection. Dose response data showed that as few as 1×10^7 IMLNC could cause a significant reduction in worm numbers when given in combination with IS. These experiments indicate that both antibodies (IS) and sensitized lymphoid cells (IMLNC) are required for effective resistance to *N. dubius*.

Keywords: *Nematospiroides dubius*, nematode, parasite, immunity, lymphocytes, antibodies, small intestine

Introduction

The parasitic nematode *Nematospiroides dubius*, like many important intestinal parasites, gives rise to a chronic primary infection and does not readily stimulate acquired immunity in its murine host (Bartlett & Ball 1974, Behnke & Wakelin 1977). It, therefore, constitutes a convenient laboratory model for the study of the factors responsible for the failure of the immune system to reject such parasites from otherwise immunocompetent animals.

Several workers have reported that resistance to *N. dubius* can be elicited only after two or more immunizing infections (Van Zandt, Cypess & Zidian 1973, Bartlett & Ball 1972, Hosier 1974) and that there is a considerable variation in the degree of resistance evoked in different strains of mice by identical immunizing procedures (Cypess & Zidian 1975, Prowse *et al.* 1979). Five distinct manifestations of immunity have been described: 1 reduction in the number of larvae surviving the tissue phase of their development, 2 arrested development of a proportion of the worms in the intestinal tissue, 3 reduction in

the size and 4 fecundity of the worms which complete development and 5 expulsion of adult worms from the host intestine (Jones & Rubin 1974, Prowse *et al.* 1979, Behnke & Parish 1979c). Although, it is possible to measure each of these effects separately the most convenient parameter and ultimately the most relevant is a quantitative measurement of the surviving adult worm population.

The expulsion of adult *N. dubius* from immune mice is a slow process when compared to the rapid loss of *Trichinella spiralis*, *Trichuris muris* or *Nippostrongylus brasiliensis* during the acute phase of the rejection of these nematodes (Behnke & Wakelin 1977, Prowse *et al.* 1979). Despite this difference there is evidence that the expulsion of adult *N. dubius*, like that of the latter species is a T cell dependant phenomenon. Prowse *et al.* (1978) demonstrated that athymic mice were incapable of acquiring resistance to *N. dubius*, but expelled the parasite when reconstituted with T cells. It has proved more difficult, however, to demonstrate the involvement of lymphoid elements in mediating resistance to *N. dubius* by passive transfer studies. Several such attempts have been recorded but these have been largely unsuccessful (Bartlett & Ball 1974, Chaicumpa, Jenkin & Rowley 1976) or at the most only partially effective (Jones & Rubin 1974, Cypess 1970). It has been suggested that *N. dubius* is more robust in tolerating conditions in the intestine sufficiently adverse to cause the loss of other species of gut parasites (Day *et al.* 1979). We have therefore attempted to devise a way of impairing the parasite in order to increase its sensitivity to immunologically mediated changes in the host intestine.

In the present report we have made use of our recent finding that partial attenuation of the infective larvae of *N. dubius* by exposure to 5 krad of Cobalt 60 irradiation before infection, produces worms which are more susceptible to the activities of immune processes than normal worms (Behnke & Parish 1979b). It is demonstrated that immunity to *N. dubius*, as monitored by worm counts following infection with irradiated larvae, can be transferred with immune serum (IS) and with immune mesenteric lymph node cells (IMLNC). However, considerably greater protection is transferred to recipient mice when they receive both IS and IMLNC.

Materials and methods

ANIMALS

Random bred CFLP mice and inbred NIH mice were used throughout this work. The animals were maintained and bred under conventional animal house conditions in the Zoology Department of Nottingham University.

Nematospiroides dubius

The origin and maintenance of our strain of *N. dubius* and the methods used for infection, recovery of worms and faecal egg counts have already been described (Behnke & Wakelin 1977, Behnke & Parish 1979c).

IMMUNIZATION OF SERUM AND CELL DONORS

The immunization procedure for serum donors has been described previously (Behnke &

Parish 1979a). Separate pools of immune serum (IS) were prepared, in CFLP mice, for all the experiments described in this paper. Control serum (CS) was obtained from naive CFLP mice. The serum was stored at -20°C in aliquots corresponding to the total volume of serum to be injected on each occasion and was thawed immediately before use.

Immune mesenteric lymph node cells (IMLNC) were obtained from groups of female NIH mice immunized by a divided primary infection (Behnke & Wakelin 1977), challenged with 150 infective larvae of *N. dubius* on day 50 and killed for cell transfer 8 days later. Control mesenteric lymph node cells (CMLNC) were obtained from naive female NIH mice of a similar age.

PROCEDURES AT CELL TRANSFER

Lymph nodes (LN) were removed into ice-cold sterile Hanks's saline. The LN were washed three times in fresh sterile saline, fatty tissue was carefully removed and a cell suspension was made by pressing the LN through a sterile stainless steel gauze (32 meshes/cm) into sterile medium 199, without glutamine, containing 2% fetal calf serum (FCS). Large cells were removed by sedimentation for 5 min. The supernatant cells washed three times in cold medium 199+FCS, counted and their viability assessed by the Trypan blue exclusion test. The required number of cells was injected intraperitoneally in 0.5 ml of suspension. Cells were transferred to recipient mice on day -5 (experiment 7), day -3 (experiments 4 and 5) or on day -4 (all remaining experiments) in relation to the day of infection.

In experiment 2, a batch of IMLNC was subjected three times to freezing and thawing immediately before injection. A second batch of IMLNC was treated with an equal volume of 0.25% glutaraldehyde in medium 199+FCS for 20 min at room temperature (22°C). The cells were then washed three times in medium 199+FCS and were left to stand for 10 min at 0°C in between each wash.

IRRADIATION OF THE INFECTIVE LARVAE OF *N. DUBIUS*

The infective, third stage larvae of *N. dubius* were exposed to gamma radiation from a Cobalt 60 source in the Chemistry Department of Nottingham University. At least 50 ml of a larval suspension were prepared and adjusted to contain the required dose of larvae in 0.2 ml of suspension. Twenty ml aliquots were then transferred to plastic universal tubes, which were placed 10 cm from the source. At this distance the rate of exposure was approximately 1 krad per min. The temperature in the exposure chamber during irradiation never exceeded 18°C and therefore the decrease in radiosensitivity which is known to occur at higher temperatures should not have been apparent in our experiments (Fitzpatrick & Mulligan, 1968). After irradiation the contents of the universal tubes were transferred to 25-ml conical flasks and a magnetic stirrer was used to ensure a uniform dispersal of larvae in the inoculum. Throughout this work, the larvae were exposed to 5 krad of irradiation. Additional control groups, infected with the same number of normal larvae were included in all experiments to monitor the reduction intake attributable to irradiation alone. A dose of 5 krad would be expected to reduce the number of *N. dubius* established by 10-30% (Behnke, Parish & Hagan 1980).

STATISTICAL ANALYSIS OF RESULTS

All the results were expressed in terms of mean worm recovery \pm s.e. and were analysed for significance by the non-parametric Wilcoxon test (Sokal & Rohlf 1969). A value of $P < 0.05$ was considered to be significant.

Results*Transfer of immunity with immune mesenteric lymph node cells and immune serum*

Several experiments were carried out in which groups of mice were given IMLNC, IS, or IMLNC + IS and were infected with 250 irradiated larvae of *N. dubius*. Worm burdens were determined at various times after infection. A representative experiment is shown in Table 1. On day -4, 1×10^8 IMLNC were injected i.p. in group C and D. Groups B and D were given 0.5 ml of IS on day 0, 1 ml on day +1, 1 ml on day +4 and 0.5 ml on day +6. The mice were killed on days 18 and 19, each group comprising four to five animals. An additional control group not shown in Table 1, infected with 250 normal larvae of *N. dubius* had 189 ± 3.2 worms indicating that the reduction in worm burden attributable to irradiation at 5 krad was of the order of 13%. The results show that IS and IMLNC when administered alone caused a significant reduction in worms (35.4% and 40.3% respectively) but a considerably greater reduction was recorded in the groups treated with both IS and IMLNC (92% protection). Essentially similar results were obtained in two subsequent experiments (not illustrated in Table 1) in which additional control groups were included. Thus, in experiment 2a IS reduced the worm burden by 21.4% ($P < 0.05$) and IMLNC by 8.4% (not significant), but when mice were given both IS and IMLNC the

Table 1. Evidence that antibodies and immune lymphoid cells are required for full expression of immunity to *Nematospiroides dubius*

Treatment	Number of worms recovered after infection with 250 irradiated <i>N. dubius</i> larvae			
	Day 18		Day 19	
	Mean \pm s.e.	% reduction	Mean \pm s.e.	% reduction
A. Control Group	164.2 \pm 5.6		154.6 \pm 7.6	
B. IS†	106.0 \pm 5.5*	35.4	103.2 \pm 6.8*	33.2
C. IMLNC‡	98.0 \pm 7.6*	40.3	75.8 \pm 8.0*	51.0
D. IS+IMLNC	13.3 \pm 4.9*	92.0	14.8 \pm 2.6*	90.4

* $P < 0.01$.

† Immune serum.

‡ Immune mesenteric lymph node cells. Statistical analysis of results. All groups were compared to the control group killed on the same day.

reduction was 83.9% ($P < 0.05$) and the few surviving worms were greatly stunted. Similarly in experiment 2b, a combination of IS and IMLNC was far more protective (90.6% reduction) than either IS or IMLNC alone (10.2% and 20.7% reduction respectively). Control serum, CMLNC and CS+CMLNC were totally without effect. Additional control groups in experiments 2a and 2b established the requirement for live IMLNC since cells killed by freezing and thawing or by treatment with glutaraldehyde did not co-operate with IS. Heat inactivated IS was as effective as untreated IS, eliminating the possibility that a heat labile component was involved.

The time course of worm expulsion in mice given IS, IMLNC and IS+IMLNC

Four separate experiments were carried out in which mice were given IS, IMLNC or IS+IMLNC and were killed in small groups on various days after infection. In experiment 5 the mice were infected with normal infective larvae of *N. dubius*, whereas in experiments 3, 4 and 6 irradiated larvae were used. The results of all four experiments are presented in Figure 1 in order to illustrate the variation in the degree of protection

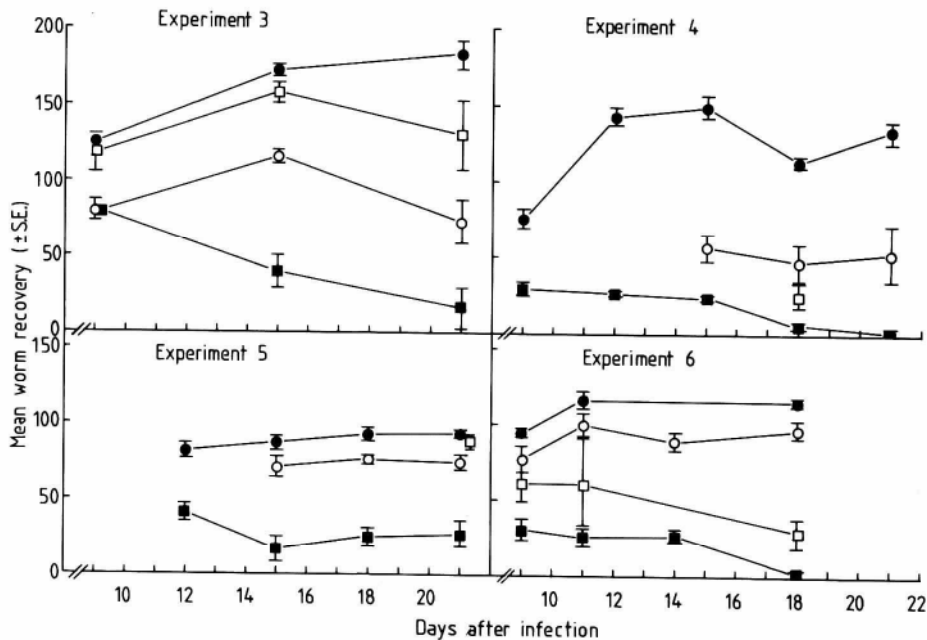


Figure 1. Expulsion of *N. dubius* from control mice (●—●), mice given IMLNC (□—□), IS (○—○) or IS+IMLNC (■—■). The mice were given 200 (experiments 4 & 6) or 250 (experiment 3) larvae irradiated with 5 krad before infection. Control groups infected with normal larvae to monitor the effect of irradiation are not shown, but the mean number of worms recovered \pm s.e. was as follows; 238.4 ± 6.6 (d21), 151.2 ± 6.2 (d18) and 129.5 ± 7.1 (d18) respectively. In experiment 5 all the mice were infected with 150 normal larvae of *N. dubius*. In all four experiments 1×10^8 IMLNC were transferred on d-4 (experiments 3 & 6) or on d-3 (experiments 4 & 5). In experiments 3, 4 & 5, IS was given as follows; 0.5 ml on d0, 1 ml on d+1, 1 ml on d+4. In experiment 6, IS was given as 0.5 mls on d0, 1 ml on d+1 and 0.5 ml on d+3. All the groups not receiving IS were injected with identical volumes of sterile saline.

transferred by IS and IMLNC. Thus, in experiment 3 and 5 IMLNC+IS co-operated synergistically in transferring immunity, the reduction in worm numbers in this group being far greater than that expected from the combined contribution of IS and IMLNC acting independently. In contrast in experiments 4 and 6 IMLNC, when administered alone, transferred considerably higher levels of protection than in experiments 3 and 5 and hence whilst the mice given IS+IMLNC had comparatively fewer worms, the enhanced protection in this latter group corresponded to no more than the additive effect of IS and IMLNC as reflected in mice given these components separately. However, it is quite clear from all four experiments that mice given both IMLNC and IS had fewer worms than any of the remaining groups after day 9. This suggests that a substantial proportion of the worms in this group was either arrested or retained in the intestinal tissue as is known to occur in immune mice. It is equally evident that the surviving worms in mice given both IS and IMLNC were rejected during the course of the experiments, the only exception being experiment 5 in which normal larvae were used at infection.

The effect of IS and IMLNC, alone and in combination, on the fecundity of the parasites is shown in Figure 2. In this experiment (data from experiment 5) IMLC did not have a major effect on egg production. However, when mice were treated with IS the eggs did not appear in the faeces until day 14. Egg output then rose considerably, although it remained lower than the control group. The delay in egg production was greatest in the final group given IS + IMLNC, where eggs were first recorded in the faeces on day 18 and were present in only very small numbers on day 21 when the experiment was terminated.

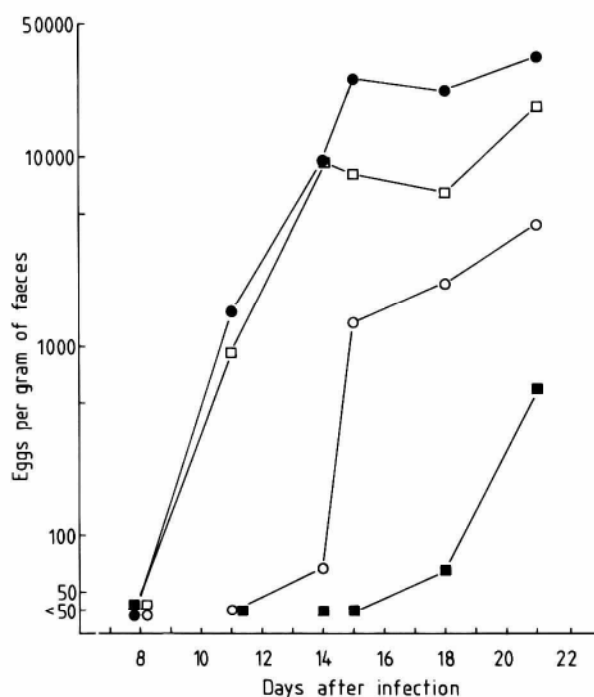


Figure 2. The mean daily worm-egg count recorded from groups of control mice (●), mice given IMLNC (□), IS (○) or IS + IMLNC (■) after an infection with 150 normal larvae of *N. dubius* on day 0 (experiment 5).

The relationship between the number of IMLNC transferred and the degree of immunity expressed in recipient mice

Figure 3 shows the results of two experiments in which groups of mice were treated with different numbers of IMLNC in order to determine the minimum number of cells required to achieve a reduction in worm burden. Both sets of data are presented because, as in the previous section, some of our experiments detected a clear synergistic effect of IS + IMLNC in mediating protection against *N. dubius* (e.g. experiment 7 at 10^7 , 5×10^7 and 1×10^8 IMLNC) whilst in others no such additional enhanced effect was evident (experiment 8). IS caused a reduction in parasite numbers although the extent of this reduction was quite different (14.7% experiment 7 and 62.7% experiment 8). When 1×10^6 – 1×10^8 IMLNC were given (experiment 7) there was approximately a 20% reduction in worm numbers compared to the groups given control cells or no treatment at all. A substantially greater effect was recorded only in the group given 2×10^8 cells. In contrast the cells used in experiment 8 were much more effective at lower doses and Figure 3b shows a negative relationship between the number of cells administered and the surviving parasite burden in recipient mice. The administration of IS + IMLNC resulted in considerably greater protection of recipients in both experiments even when low numbers of cells were transferred. Thus 1×10^7 IMLNC caused a significant reduction

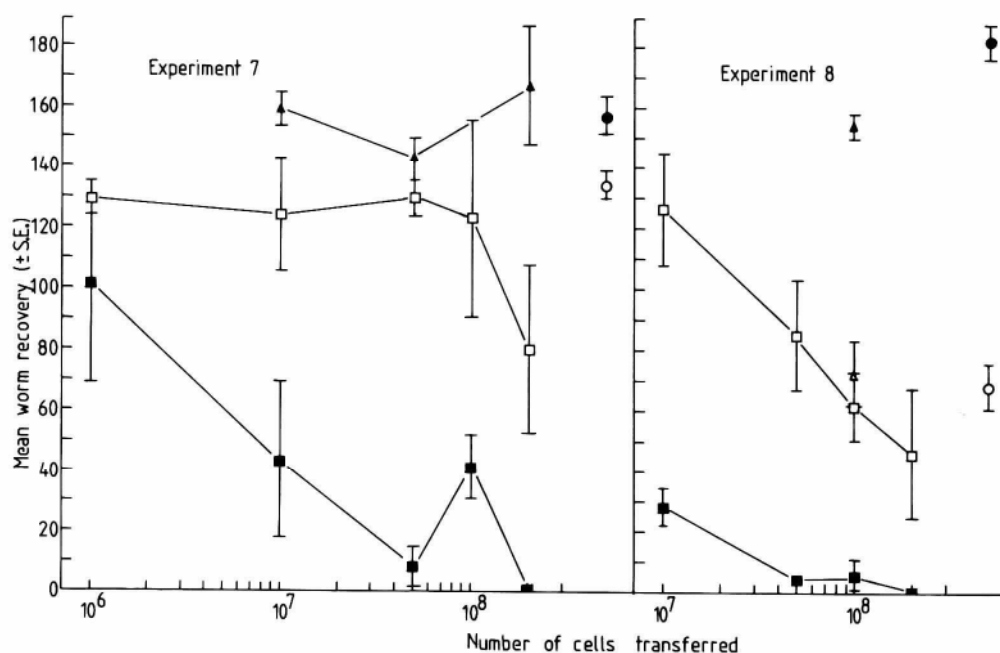


Figure 3. The relationship between the number of IMLNC transferred and the degree of immunity expressed in recipient mice. Groups of 5–6 mice were infected with 200 irradiated larvae of *N. dubius* on d0 and were killed on d18 for worm counts. In experiment 7, IS was given as follows: 0.5 ml on d0, 1 ml on d + 1 and 0.5 ml on d + 3. In experiment 8, IS was given: 0.5 ml on d0, 1 ml on d1 & d4. In both experiments all the groups not receiving IS were injected with the same volume of sterile saline. IMLNC were injected i.p. in 0.5 ml of suspension on d – 5 (experiment 7) and d – 4 (experiment 8). Control group (●); Control cells (▲); IS (○); control cells + IS (△); IMLNC (□); IMLNC + IS (■).

and >90% protection was recorded when 5×10^7 IMLNC were used. However, the additional effect reflecting a synergistic interaction between IS and IMLNC was seen only in experiment 7.

Discussion

The mechanism of host resistance to infectious organisms is most conveniently studied by passive and adoptive transfer methodology, where the exact role and requirement for specific components can be assessed. In the case of *N. dubius* it has been known for some years that acquired immunity can be elicited in mice after several immunizing infections (Van Zandt 1961), but success in attempting to analyse the mechanism by passive transfer has been rarely achieved and is poorly documented. In the present work we have monitored the activity of the immune system against radiation attenuated worms, since it has been shown that irradiated worms are more susceptible to the immune response than normal worms, thereby providing a more sensitive experimental assay for functional immunity in the host (Behnke & Parish 1979b). Our results provide clear evidence that lymphoid cells instrumental in mediating resistance are present in the mesenteric lymph nodes of immune mice and that antibodies in the serum of resistant mice have a crucial role in immunity to *N. dubius*. However, it is equally evident that IMLNC were only able to exert a rapid and marked effect on parasite numbers when the host was simultaneously treated with IS. Neither of the two components were capable of mediating a comparable effect when administered alone.

There was a considerable variation in the activity of the different pools of IMLNC; some induced high levels of protection, whereas others were only effective when very large numbers of cells were transferred. Similarly the different pools of IS transferred variable levels of resistance (e.g. experiment 7—14.7%, experiment 8—62.7%). In mice given both IMLNC and IS there was a greatly enhanced early loss phase, presumably associated with the retention of developing or arrested worms in the intestinal tissue, and then the surviving worms were slowly rejected during the second and third weeks of infection. However, these results cannot be explained simply by an additive effect of the two components. In experiment 7 (Figure 3) for instance, IS gave 14.7% protection, 5×10^7 IMLNC gave 17.6% protection, but mice given both IS and 5×10^7 IMLNC were protected by 94.6%. This result is supported by additional data in Table 1, indicating that co-operation between the two components lead to a greatly enhanced effect against the parasite.

The mechanism of this synergistic effect is not known at the present time but pertinent in this respect is the recent proposal that *N. dubius* survives by interfering with the host's ability to generate and express an effective anti-worm response in the intestine (Behnke, Wakelin & Wilson 1978, Jenkins & Behnke 1977). It is possible that in addition to a direct anti-parasite effect, antibodies in IS neutralized the parasite factors exerting this immunodepressive effect and protected the host's immune system allowing the second component mediated via the transferred IMLNC and the recipient's own lymphoid cells to become operational. The mesenteric lymph nodes would be expected to contain substantial numbers of plasma cells and B lymphocytes and transferred cells may have contributed to levels of protective antibody in recipient mice, as also to the concentration of local intestinal antibody. However, whilst a role for IgA secreting plasma cells, as this

second component has not been discounted (Despommier *et al.* 1977), a more probable explanation is that non-antibody producing cells (T lymphocytes) were involved (Prowse *et al.* 1978).

Although these results indicate that co-operation between lymphocytes and antibodies is essential for full expression of immunity to *N. dubius*, they do not clarify how worm loss was brought about in recipient mice. It is significant, however, that there was a substantial reduction in the number of worms surviving the tissue phase of infection (Figure 1) in these animals and it is therefore probable that the co-operative effect of IS+IMLNC was essentially directed at trapping and destroying the worms within the intestinal walls. It is well established that cellular granulomata form around the sites of development of the parasite, especially in immune animals, and play an important role in mediating resistance to re-infection (Prowse *et al.* 1979). The stunted nature of the worms which survived the tissue phase may have been the consequence of antibody mediated impairment of development in the gut tissues. These worms were lost during the second phase of expulsion, from the gut lumen which was accompanied by gross inflammatory changes in the intestine and it could be that the mechanism involved was not dissimilar to that reported for other intestinal helminth parasites which are expelled by the host (Ogilvie & Love 1974, Wakelin & Lloyd 1976, Rothwell & Griffiths 1977, Wakelin 1978). However, this possibility will be resolved only when the components involved in transferring effective resistance have been fully characterized and this information, in turn, should help to elucidate how *N. dubius*, like many more important parasitic nematodes, avoids being rejected by the host during a normal primary infection.

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