***Nematospiroides dubius* in the mouse: evidence that adult worms depress the expression of homologous immunity**

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Summary Mice immunized by a single infection with irradiated (25 krad) larvae of *N. dubius* were very resistant to subsequent challenge. However, when normal larvae were administered together with irradiated larvae at immunization, the acquired immunity expressed against a challenge infection was markedly depressed. It was found that as few as 50 normal *N. dubius* larvae interfered with the immunity that would have otherwise been elicited by the concurrently administered irradiated larvae, but this depressed response was totally alleviated when the normal worms were removed after completing their development in the intestinal mucosa and before they reached adulthood. Adult *N. dubius* were transplanted directly into the intestines of mice either 7 days before or after immunization by irradiated larvae; it was shown that the recipient mice were less resistant to challenge than mice which had been sham operated. Transplanted adult worms themselves stimulated very little resistance to challenge in recipient mice. These results established that adult parasites are capable of depressing the expression of homologous immunity in the mouse. The possible mechanisms by which *N. dubius* might modulate the host's immunological activity at the intestinal level are discussed and it is proposed that this mechanism is of benefit to the parasite in preventing the host from eliminating the worms during a chronic primary function.

Keywords: *N. dubius*, homologous immunity, immunosuppression, immunity to irradiated larvae

Introduction

The long-term survival of gastrointestinal and tissue dwelling parasitic nematodes in their mammalian hosts is a phenomenon for which there is, as yet, no satisfactory immunological explanation. By definition, the organisms must either fail to initiate or alternatively must evade/resist potentially host protective immune responses. Most nematodes undergo several distinct developmental stages in their definitive hosts and there is ample evidence that in some species (e.g. *Trichinella spiralis*—Philipp, Parkhouse & Ogilvie 1980, Bell, McGregor & Despommier 1979) immunity is stage specific. This raises

the possibility that immunity, elicited in the host by developing larvae, may be ineffective against the adult parasites. In the case of *Nematospiroides dubius*, immunity to reinfection is known to be stimulated by the larval stages, but transplanted adult worms have been shown to survive for many weeks in mice totally immune to larval challenge (Williams 1982). However, other workers have found that adult worms developing from a larval infection in SJL/J female mice (Mitchell 1979) or from a challenge infection in immune mice (Behnke & Wakelin 1977, Day *et al.* 1979) are lost from the intestine, implying that under these circumstances anti-adult worm host protective responses can occur.

An alternative explanation for the chronic survival of adult *N. dubius* is that the parasite itself affects the host's ability to generate and express an effective anti-parasite response in the intestine and there is evidence to support this hypothesis. A primary infection with *N. dubius* is known to severely depress the host's immune response to concurrently administered un-related antigens such as sheep erythrocytes (SRBC) (Shimp, Crandall & Crandall 1975, Ali & Behnke 1982), bacterial lipopolysaccharide (LPS) and oxazalone. Ali & Behnke (1983) suggested that at least a proportion of the immunodepression observed in response to parenteral challenge with SRBC, may be a necessary consequence of the parasite's survival mechanism, which presumably operates more potently at the intestinal level. *N. dubius* exerts a marked depressive effect on the host's ability to expel a concurrent infection with *T. spiralis* (Behnke, Wakelin & Wilson 1978), an observation which may be explained by the parasite exerting an anti-inflammatory effect in the intestine (Hagan & Wakelin 1982). Other workers have shown that concurrently infected mice produce diminished responses to *T. muris*, *H. diminuta* and *N. brasiliensis* (Jenkins & Behnke 1977, Hopkins 1980, Jenkins 1975). This strongly suggests that local intestinal immunological events are influenced by *N. dubius*, possibly to enhance its own survival. The experiments reported in this paper were specifically designed to determine whether immunodepression of the host during infection with this parasite would be of benefit to *N. dubius* itself.

Materials and methods

Mice

Male and female randomly bred CFLP mice were bred and maintained under conventional animal house conditions in this department. The animals were used when at least 6-weeks-old.

Nematospiroides dubius

The methods used to maintain *N. dubius*, infect mice and recover worms have all been described previously (Jenkins & Behnke 1977). The infective larvae of *N. dubius* were irradiated by the methods described by Behnke, Parish & Hagan (1980).

In some experiments, animals were infected with adult *N. dubius* which were introduced directly into the small intestine. The mice were anaesthetized with sodium pentobarbitone (Sagatal, May & Baker, Dagenham), shaved and cleansed with 70% alcohol. A 1 cm incision was made to the right of the midline and approximately above the junction of the duodenum with the stomach. The proximal duodenum was extracted and

punctured with a cannula (medicut, 1.7 mm o.d., intravenous cannula, Sherwood Medical Industries). The hypodermic needle was then withdrawn, leaving the plastic sheath in place. Adult worms were washed several times in sterile saline, taken up into a syringe in 0.3 ml of saline and injected through the plastic sheath into the duodenum. The sheath was removed and the wound closed with a single stitch (0.7 metric, Mersilk mersuture, Ethicon, Edinburgh). The exposed intestine and peritoneal cavity were sprayed with an antibiotic mixture of bacitracin, polymixin and neomycin (Rikospray, Riker Laboratories, Loughborough) and the intestine was replaced. The body wall was closed with two sutures (0.1 metric), sprayed with antibiotic and the skin was closed with two or three stitches, cleansed with 70% alcohol, and protected with a covering of plastic skin (Nobecutane, Astra Chemicals, Watford).

Anthelmintic

Pyrantel embonate (strongid P paste, Pfizer) was used to remove adult *N. dubius* from infected mice. A dose of 100 mg/kg was administered orally as an aqueous suspension. This dose level is adequate for the removal of all adult worms from the intestinal lumen (Behnke & Wakelin 1977).

Statistical analysis of results

The mean number of worms recovered (MWR) from infected groups is expressed as a percentage of the control value ± 1 standard error (1 s.e.) with the control represented as 100%. Actual counts ± 1 s.e. are included in the text for reference. Results were analysed for significance using the non-parametric Wilcoxon test (Sokal & Rohlf 1969). A value of $P < 0.05$ was considered to be significant.

Results

STIMULATION OF IMMUNITY TO *N. DUBIUS* BY CONCURRENT INFECTION WITH NORMAL AND IRRADIATED LARVAE

Heavily irradiated (25 krad) infective larvae of *N. dubius* do not develop to maturity and most are lost within 5 weeks of infection (Behnke, Parish & Hagan 1980). Nevertheless, such larvae stimulate strong resistance to reinfection in the mouse (Hagan, Behnke & Parish 1981). These two findings were exploited in the present work in order to immunize mice effectively by a single infection, without exposing the animals to adult worms.

In the first experiment, groups of female mice were immunized by infection with 200 normal (group B), 200 irradiated (group D) or both 200 normal and 200 irradiated larvae (group C) of *N. dubius*. The worms were removed by treatment with pyrantel on day 35, challenged on day 37, and killed 2 weeks later. Worm recoveries were compared with those of a control group (group A) and are shown in Figure 1.

Mice which had been exposed to a normal primary infection showed little resistance against challenge (B vs A, $P = \text{N.S.}$), whereas animals immunized with irradiated larvae were very resistant (D vs A, $P = 0.01$) and harboured only 3.0 ± 1.4 worms. However, group C which had been given both normal and irradiated larvae on day 0 had a

Figure 1. Stimulation of immunity to *N. dubius* by concurrent infection with normal and irradiated larvae. The figure shows the number of worms recovered after a challenge infection with 200 normal larvae of *N. dubius*. The control group A, had a MWR of 150.6 ± 6.7 . Groups B–D were treated as follows; B ($n=6$) was given 200 normal larvae on day 0; C ($n=5$) was given 200 normal and 200 irradiated larvae; D ($n=4$) was given 200 irradiated larvae alone. On day 35 all the groups were treated with pyrantel. The challenge infection was given on day 37 and the mice were killed 14 days later. Additional groups of mice were killed to monitor the immunizing infection. The MWR was as follows; group B ($n=3$)— 104 ± 8.5 ; group C ($n=3$)— 105.7 ± 3.7 ; group D ($n=3$)— 1.3 ± 0.7 .

substantial worm burden (52% of the control group; C vs A, $P=0.01$; C vs D, $P=0.01$) after challenge. This result suggested that the normal infection exerted a deleterious effect on the immunity which would otherwise have been stimulated by the irradiated larvae in the doubly infected mice.

THE NUMBER OF NORMAL LARVAE REQUIRED TO EFFECTIVELY INTERFERE WITH IMMUNITY STIMULATED BY IRRADIATED LARVAE

The second experiment was carried out to determine whether different numbers of normal larvae would exert a varying effect on the extent of the depression in resistance observed in mice immunized by concurrent infection with both normal and irradiated larvae. The experimental design was similar to that used in the preceding experiment, except that

Table 1. The number of normal larvae required to effectively interfere with immunity stimulated by irradiated larvae. The table showing the MWR after the challenge infection

Group	Treatment	No. mice	Mean worm recovery $\pm$ s.e.	Statistical analysis	
				v.A	v.B
A	—	6	77.3 ± 4.5	—	—
B	200I	6	9.5 ± 8.1	$P < 0.005$	—
C	200I + 50N	6	49.0 ± 9.0	$P = 0.01$	$P = 0.01$
D	200I + 150N	5	53.0 ± 12.0	$P = \text{n.s.}$	$P = 0.01$
E	200I + 300N	6	57.0 ± 5.3	$P < 0.025$	$P < 0.005$
F	50N	6	72.5 ± 3.8	$P = \text{n.s.}$	$P < 0.005$
G	150N	6	65.2 ± 6.1	$P = \text{n.s.}$	$P < 0.005$
H	300N	6	70.2 ± 6.4	$P = \text{n.s.}$	$P < 0.005$

Infections were given on day 0 and infectivity control groups were killed on day 14. All the mice were given anthelmintic on day 28 and 30 and were challenged with 100 normal larvae on day 35. Twenty-three days later the mice were killed and their worms were recovered. Additional groups of mice were killed to monitor the infectivity of both immunizing and normal infection. The MWR for these infectivity controls was as follows: group B ($n=4$)—0; group F ($n=4$)— 54.5 ± 3.6 ; group G ($n=4$)— 76.8 ± 3.8 ; group H ($n=4$)— 153.0 ± 5.6 . I = Irradiated (25 krad) larvae; N = normal larvae; n.s. = not significant.

additional groups were included, these being infected with 50, 150 or 300 normal larvae of *N. dubius* either alone or in conjunction with 200 irradiated larvae (Table 1). Despite some lack of infectivity in the immunizing inocula (groups G & H), a range of infection levels was established. At these levels of infection, normal larvae did not stimulate immunity to challenge, but all three infection levels successfully impaired acquired immunity in mice concurrently immunized with 200 irradiated larvae (groups C, D, E all significantly higher than group B). Although group E, given 300 normal larvae had the highest worm burden following challenge infection, even group C, given only 50 normal larvae, was still less resistant than the mice immunized by irradiated larvae alone (group B).

THE EFFECT OF REMOVING DEVELOPING WORMS IN MICE GIVEN BOTH NORMAL AND IRRADIATED LARVAE BEFORE MATURITY

This experiment was similarly designed to experiment one, except that an additional group, which was treated with pyrantel on days 7–11, was included. The purpose of the anthelmintic was to remove all the developing worms as they returned to the gut lumen after completing their development in the intestinal mucosa. As recorded in the legend to Figure 2, 1.7 worms were recovered from this group. The MWR following challenge infection are shown in Figure 2 and confirm that as in previous experiments normal larvae

Figure 2. The effect of removing developing worms before maturity in mice given both normal and irradiated larvae. The figure shows the number of worms recovered after a challenge infection with 200 normal larvae of *N. dubius*. The control group (A, $n=6$) had a MWR of 101.2 ± 3.4 . Groups B-E were treated as follows; B ($n=6$) was given 200 normal larvae on day 0; C ($n=6$) was given 200 normal and 200 irradiated larvae; D ($n=6$) was given 200 normal and 200 irradiated larvae, but also treated with pyrantel on days 7-11; E ($n=4$) was given 200 irradiated larvae alone. On days 28 and 30 all the groups were treated with pyrantel. The challenge infection was given on day 32 and the mice were autopsied 14 days later. Additional groups of mice were killed to monitor the immunizing infection. The MWR was as follows: group B ($n=3$)— 131 ± 3.9 ; group C ($n=3$)— 127.3 ± 6.8 ; group D ($n=3$)— 1.7 ± 0.3 ; group E ($n=3$)— 7.3 ± 2.6 .

did not stimulate significant levels of immunity (group B), irradiated larvae were highly immunogenic (group E) and that mice given both normal and irradiated larvae (group C), were less resistant to challenge than mice given irradiated larvae alone (group E). However, group D, which in addition to infection with normal and irradiated larvae, was treated with an anthelmintic drug to remove all the worms before patency, eliminated 86.4% of the challenge infection. This experiment indicated that the removal of the parasite prior to adulthood was crucial to the effective expression of acquired immunity, but it did not make clear whether the high levels of immunity seen in this group resulted from the irradiated larvae or from the 9-11 days infected with normal worms. The latter is

Figure 3. Depression of immunity by adult worms in mice immunized with irradiated larvae. The figure shows the number of worms recovered after a challenge infection with 200 normal larvae of *N. dubius*. The results were compiled from three separate experiments in which the control groups A had the following MWR: 91.5 ± 4.8 ($n=6$), 111.7 ± 6.1 ($n=7$), 151.8 ± 7.2 ($n=6$). As shown above groups C–G all received 200 irradiated larvae of *N. dubius* on day 0. In order to confirm the infectivity of the immunizing inocula and the effect of irradiation on larval survival, additional groups of mice in each of the three experiments were infected with larvae before and after irradiation. The MWR from groups infected before irradiation were 116.2 ± 6.7 , 51.4 ± 2.7 , 135.3 ± 7.5 and the corresponding MWR from mice given irradiated larvae were 11.8 ± 1.9 , 1.2 ± 2.7 , 0, respectively. Groups B and E received 100 normal adult worms by laparotomy on day -7 or day +7 respectively. Groups D and F were sham operated on the same days. All the mice received anthelmintic on day 42 and 44 and were challenged with 200 normal larvae on day 49. The mice were killed and their worms recovered on day 77, 4 weeks after challenge.

a realistic possibility since it is known that immunizing infections terminated before patency are more immunogenic than those of longer duration (Behnke & Hannah 1983).

DEPRESSION OF IMMUNITY BY ADULT WORMS IN MICE IMMUNIZED WITH IRRADIATED LARVAE

In order to establish whether it is the adult *N. dubius* which impair immunity in mice concurrently infected with normal and irradiated larvae, the following experiments were carried out. Groups of mice were infected with 200 irradiated larvae on day 0 and received 100 normal adult worms by laparotomy either 7 days before or after the irradiated larval

infection. The establishment of transplanted worms was confirmed by faecal egg counts and the worms were removed by treatment with anthelmintic on day 42. Immunity against *N. dubius* was assessed against a challenge infection with normal infective larvae. Three such experiments were carried out and since the results from all three were similar, the pooled results are presented in Figure 3.

It can be seen that as in previous experiments 25 krad irradiated larvae elicited >90% protection against challenge (group G) and that this resistance was not impaired by sham transplantation on day -7 (group D) or day +7 (group F). However, both groups of mice into which adult worms were transplanted (groups C & D) were less resistant to challenge (C vs D, $P < 0.001$; E vs F, $P < 0.0001$). There was also a significant difference between group C and E ($P < 0.005$) suggesting that transplantation of adult worms before the immunizing infection was more effective in depressing immunity than the same treatment after immunization. Interestingly, mice receiving transplanted worms alone (group B), had marginally fewer worms ($P < 0.05$) following the challenge infection than the control group (A), indicating that a 7-week-period of infection with transplanted adult worms elicited very little immunity to subsequent larval challenge.

Discussion

The experiments presented here leave little doubt that adult *N. dubius* exert a profound depressive effect on the host's ability to respond to a homologous immunizing infection. Non-specific immunodepression of the host is a well-established phenomenon in parasite immunology (Wedderburn 1974, Ogilvie & Wilson 1977), but its precise value as a survival strategy for the parasites involved remains controversial. In most species it has proven difficult to show that there is any benefit for the parasite. Indeed, MacAskill *et al.* (1981) demonstrated that the response of mice infected with *Trypanosoma brucei* to trypanosome antigens is unaffected by concurrent immunodepression of the host. Furthermore, the course of infection with *Plasmodium berghei* in mice is independent of parasite induced immunosuppression since abrogation of the suppressor cell population, which is active in this model, and the consequent restoration of a normal response to oxazalone had no effect on the course of parasitaemia nor on parasite survival *in vivo* (Lelchuk, Sprott & Playfair 1981). However, other studies have shown that the presence of established parasites may increase the host's susceptibility to further homologous infection (*Brugia pahangi* in jirds, (Klei, McCall & Malone 1980)), but it is possible that in *B. pahangi*, parasite induced physiological alterations of local tissues and not immunological events contributed to the observed effects. We consider that such an explanation is unsatisfactory for our own results and that the work reported here has established that in the case of *N. dubius*, interference by the parasite with immunological events at the intestinal level contributes to the host's failure to control challenge infections. It may also be the underlying mechanism for the chronic primary infections experienced with this organism.

Evidence to support our hypothesis is available from other studies. Hagan & Wakelin (1982) have demonstrated that in mice concurrently infected with *Trichinella spiralis* and *N. dubius*, the migration of lymphocytes to the intestine (normally greatly enhanced in mice infected with *T. spiralis* alone) is depressed, almost to the level of control uninfected mice. The authors proposed that the prolonged survival of *T. spiralis* in *N. dubius* infected mice was attributable to the reduced acute inflammatory component of the host's

response in the gut. If adult *N. dubius* are capable of secreting immunomodulatory factors (IMF) as these experiments suggest, this could explain the poor resistance in mice immunized with a single patent primary infection (Behnke & Wakelin 1977, Liu 1966, Bartlett & Ball 1974). However, in responder strains of mice (e.g. NIH), infections terminated immediately after the emergence of the larval stages from the intestinal mucosa, i.e., prior to adulthood, or infections with irradiated larvae, have been shown to be strongly immunogenic (Hosier & Feller 1973, Ey, Prowse & Jenkin 1981, Behnke & Hannah 1983, Hagan, Behnke & Parish 1981). Therefore in the absence of adult worms and presumably adult worm IMF, larval antigens elicit potent immunity in the host. In immune mice larval *N. dubius* are thought to be destroyed within mucosal granulomata by specific IgG₁ antibody, co-operating with eosinophils and possibly other cell types (Prowse *et al.* 1978, Jones & Rubin 1974, Pritchard *et al.* 1983). However, even in immune mice larvae have been shown to survive in an arrested form for long periods of time within the granulomatous intestine (Behnke & Parish 1979) and therefore some immunodepressive activity by the larval parasite is a further possibility (see also Jenkins & Behnke 1977).

Whatever the mechanism operating against the tissue stages of *N. dubius*, adult worms would need to be expelled by qualitatively distinct components of the effector mechanism because of their position in the gut lumen. In fact, some strains of mice reject adult worms (Mitchell 1979), as also do abnormal hosts such as the jird (Hannah & Behnke 1982), and there is evidence that the mouse can generate within its intestine an environment which is detrimental to the parasites' survival. We have observed that mice injected with a low-level infection of *N. dubius* (100 worms) when challenged with a moderate to heavy inoculum of *T. spiralis* (250+ worms), lose a proportion of their adult *N. dubius*, despite the delayed and partially depressed host response to *T. spiralis*. Nevertheless, some *N. dubius* survive even under these circumstances, implying that the adult stages of this parasite are to some extent resistant to the host's intestinal inflammatory response; a hypothesis which has already been put forward by Day *et al.* (1979). However, in view of the present work, an alternative explanation may be that even under these circumstances, the surviving worms reside in 'pockets' within the intestine where their own mechanism for inactivating the host's immune response has created areas of relative safety. It is a well-established fact that adult *N. dubius* are seldom to be found evenly distributed within the intestine. More often the worms aggregate into pockets which may contain very many worms, especially in hosts partially resistant to the parasite (Lewis & Bryant 1976).

Our findings suggest that in order to exert a full host protective response, the mouse would not only need to be sensitized to the parasites' functional antigens but would also need to be capable of neutralizing the parasites' own IMF. It is not surprising, therefore, that full term primary infections with adult *N. dubius* have proven to be of little value in promoting host resistance. Presumably, under the repeated infection regimes (Behnke & Wakelin 1977, Prowse *et al.* 1979) the host eventually overcomes parasite IMF, whereas irradiated and normal larval infections terminated before patency lead to adequate sensitization due to the absence of potentially immunosuppressive adults. The presence of IMF in the parasite's excretory/secretory (ES) products could explain the relative ineffectiveness of ES products as immunogens, despite the presence of antigens recognizable by protective antibody (Day *et al.* 1979, Hurley, Day & Mitchell 1980, Pritchard *et al.* 1983). If ES products contain IMF, one might expect their immunogenic properties to be overshadowed by the former.

Whilst our results have clearly demonstrated that *N. dubius* is capable of modulating host immunity, they have not explained how possible immunomodulatory factors may be exerting such an effect on the host. In contrast to *Fasciola hepatica*, *N. dubius* ES products are not directly toxic to murine lymphocytes (Goose 1978, Behnke unpublished observations) so their effect must be achieved by an alternative process. Primary infections with *N. dubius* elicit an IgG₁ hypergammaglobulinaemia in the host and it could be that this is achieved by IMF. Mitchell (1979) has suggested that primary infection IgG₁ blocks host-protective responses, but we have found that the elevated IgG₁ levels which occur following primary infection do not interfere with immunity transferred by concurrently injected immune mesenteric lymph node cells (Williams & Behnke 1982). However, primary infection serum does recognize adult worms and it is conceivable that IgG₁-ES immune complexes activate suppressor T cells (Mitchell 1979); a possibility which is being investigated. IgG₁ hypergammaglobulinaemia is known to be caused by continuous exposure to antigen such as would occur in infected animals (Chapman *et al.* 1979) and therefore a further possibility is that in mice infected with *N. dubius*, IgG₁ hypergammaglobulinaemia is the consequence and not the cause of the chronic infection. Purification of primary infection IgG₁ and further analysis of its biological properties should resolve this point.

Finally, our experiments have shown that at least in this model system, specific depression by the parasite of potentially host-protective responses enables the organism to survive in its host. There are many biological similarities between *N. dubius* and other more important nematode parasites, not least of which is the chronicity of primary infections and the inability of the host to develop acquired immunity rapidly. It is a real possibility that *N. dubius* is not alone in promoting its own survival within the host by immunomodulation and if this is confirmed, it will need to be carefully considered during the development of appropriate vaccines in the future.

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