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Nematospiroides dubius: factors affecting the primary response to SRBC in infected mice

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ABSTRACT

Mice infected with *Nematospiroides dubius* were incapable of responding normally to i.p. or i.v. challenge with SRBC. The HA and PFC response to SRBC in infected animals was characterized by a severe depression of antibody to SRBC on day 4 and a reduced HA peak titre during the following week. The greatest depression of the response to SRBC was associated with an interval of 14 days between infection and the administration of antigen, suggesting that a particular stage of the parasite contributed significantly to immunodepression during this critical period. It was proposed that a combination of parasite induced damage to the intestine, release of parasite secretory/excretory products and loss of appetite by the host produced trauma during which the host was incapable of responding normally.

However, mice given low-level and long-standing infections also showed reduced responses to SRBC, although these animals were not severely depressed. It is possible that this generalized weakening of host immunocompetence is the inevitable consequence of a parasite mechanism which operates more specifically to suppress the expression of homologous immunity at the intestinal level.

INTRODUCTION

It has long been known that some species of parasites cause non-specific immunodepression in their hosts, but the precise significance of immunodepression of infected hosts to the biology of the parasites involved is not understood. It has been proposed that this may be a mechanism by which the organisms facilitate their own survival (OGILVIE & WILSON, 1976) and, therefore, parasites which cause chronic infections in their hosts are of particular interest in this context.

Nematospiroides dubius is a gastro-intestinal nematode of the mouse which survives for eight or more months in a primary infection (EHRENFORD, 1954; WILLIAMS *et al.*, 1982). How the parasite evades host immunity is not known but in recent years evidence has accumulated to suggest that the parasite severely affects the host's ability to generate and express immune responses to heterologous antigens (SHIMP *et al.*, 1975; JENKINS & BEHNKE, 1977; JENKINS, 1975; CHOWANIEC *et al.*, 1972). Some studies have shown that immunodepression at the intestinal level is particularly marked in mice infected with *N. dubius* (JENKINS & BEHNKE, 1977; HAGAN & WAKELIN, 1982). For example, the survival of *Trichinella spiralis* in mice harbouring *N. dubius* was greatly prolonged (BEHNKE *et al.*, 1978). Expulsion of *T. spiralis* in doubly infected mice began later than in control animals and a residual population of worms persisted for four or more weeks after the total rejection of worms from control mice. It was suggested that the survival of *T. spiralis* in these mice was facilitated by *N. dubius* as part of its own survival strategy.

More recently HANNAH *et al.* (1982) have demonstrated that *N. dubius* interferes with the host's ability to express homologous immunity. Thus when mice were infected with normal and radiation-attenuated *N. dubius* larvae, they developed significantly less resistance against a subsequent challenge infection than a control group given radiation-attenuated larvae alone. These results support the hypothesis that immunodepression during infection with *N. dubius* is of some functional significance to

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the parasite and hence it seemed opportune at this time to re-examine the evidence for and the extent of non-specific immunodepression of responses to conventional antigens such as sheep red blood cells (SRBC), lipopolysaccharide and oxazalone in mice infected with this nematode. We here report the results of experiments studying some of the factors which influence the response to SRBC in infected animals, as a baseline on which to design more analytical studies in the future.

MATERIALS AND METHODS

Animals and experimental infections

Inbred NIH and randomly bred CFLP mice were maintained under conventional conditions in the departmental animal house. The animals were used for experiments when five to 12 weeks old. NIH mice are regarded as strong and CFLP mice moderate responders to infection with *N. dubius*. Both strains respond very similarly to SRBC and are affected in the same way by concurrent infection with *N. dubius*. Although mice of one or other strain were used in individual experiments, most experiments were duplicated in both strains.

The infective third-stage larvae of *N. dubius* were given orally by stomach tube, with the required number of larvae in 0·2 ml of aqueous suspension. The methods used to maintain *N. dubius* and to recover adult worms have been described previously (JENKINS & BEHNKE, 1977).

Immunization of mice and determination of haemagglutinating antibody titre

Sheep red blood cells (SRBC) in Alsever's solution (School of Agriculture, Animal Breeding Unit at Sutton Bonnington, University of Nottingham) were washed three times in phosphate buffered saline (PBS) and were resuspended to the required concentration. Mice were injected i.p. or i.v. with 0·3 ml of a cell suspension. Haemagglutinating antibody (HA) responses were determined by serial doubling dilution of serum on microtitre plates, the first well in each titration containing a one in four dilution of serum in PBS. 2% (v/v) washed SRBC in a volume of 0·025 ml were added to each well, and the plates were incubated at 37°C for two hours for the determination of the end point.

2-Mercaptoethanol (2ME) sensitive antibody (IgM) was determined by the following method: 10 μl of serum was diluted one in four in PBS containing 0·2 M 2ME. The plates were incubated at 37°C for 30 min and were then titrated our before addition of SRBC.

Determination of plaque forming cells (PFC)

PFC assay chambers were prepared on slides by a modified method of CUNNINGHAM & SZENBERG (1968) as described by MARBROOK *et al.* (1980). Spleen cell suspensions were prepared from individual mice on fine mesh steel grids. The cells were washed three times and suspended in a Hank's balanced salt solution (HBSS) and kept at 0°C until required. Cell viability was determined by trypan blue exclusion and the cell suspensions were usually found to be 90 to 95% viable.

Indicator cells were prepared as follows: 0·5 ml of 10% (v/v) washed SRBC were added to 2 ml of HBSS and 0·3 ml of guinea-pig serum (one in 10) as a source of complement and were kept at 0°C until required. 0·2 ml of spleen cell suspension was mixed with an equal volume of indicator cells and incubated for 10 min at room temperature. 40 μl of this mixture were delivered into each chamber, the edges of the chambers were sealed with a heated paraffin-vaseline mixture and the slides were incubated for one hour at 37°C. PFC were counted using a microscope and were recorded as PFC/spleen or PFC/10^6 spleen cells.

Statistical analysis of results

Unless otherwise stated groups of four to six mice were used throughout this work and the results are expressed as the group mean ± S.E. Where S.E.s on the Figs. overlap, only one S.E. is shown or alternatively the S.E. bar of one group is shown slightly to one side of the others. Statistical significance was determined by the non-parametric Wilcoxon test (SOKAL & ROHLF, 1969). A value of $p < 0.05$ was considered to be significant.

RESULTS

Several preliminary experiments were carried out in order to determine the optimum dose of SRBC which would permit the detection of any depression in the HA response of infected mice. The experiments covered a range of dose levels from 2×10^5 to 5×10^8 and it was concluded that a 2 to 5×10^7 SRBC gave the greatest difference between control and infected groups and therefore this dose of SRBC was used throughout the experiments reported in this paper.

The relative timing of infection with N. dubius *and administration of SRBC*

A number of experiments were carried out to determine the significance of the relative timing of presentation of antigen during infection with *N. dubius*. The results of three such experiments in which different intervals between infection and injection of SRBC were considered, are summarized in Tables I and II and Fig. 1. The HA response in mice infected for 14 or 28 days was 64 to 100% depressed four days after injection of SRBC, but the extent of the depression was reduced as the duration between infection and antigen administration decreased. When mice were infected with *N. dubius* four or eight days after SRBC, there was no significant effect on the pattern of the HA response

TABLE I. The relationship between the relative time of infection with *N. dubius* and the administration of SRBC

Group	Day on which mice were given *N. dubius**	HA response on days after administration of SRBC						
		0	4	8	12	16	20	24
A	No *N. dubius*	<3	7·7±0·2	9·0±0·3	9·7±0·3	8·2±0·2	6·4±0·2	6·0±0·3
B	*N. dubius* on d-14	<3	4·0±0·3	5·5±0·4	7·0±0·3	7·6±0·4	6·6±0·2	6·0±0·3
C	*N. dubius* on d0	<3	6·5±0·2	8·2±0·2	8·5±0·2	7·6±0·4	5·8±0·4	6·0±0·3
D	*N. dubius* on d+4	<3	7·7±0·2	8·2±0·4	8·7±0·2	7·6±0·2	6·2±0·2	6·8±0·4
E	*N. dubius* on d+8	<3	7·8±0·2	9·0±0·3	9·2±0·3	7·8±0·2	5·8±0·4	5·8±0·2

*Groups of 6 CFLP female mice were given 450 larvae on the day shown. The MWR were as follows: B, 385·0±4·0; C, 467·5±4·5; D, 420·0±23·0; E, 381·5±6·5.

TABLE II. Direct PFC to SRBC in mice infected with *N. dubius* for various periods of time

Group	No.* Mice	Treatment		Mean PFC/spleen ±S.E.	% Reduction	Mean PFC/10^6 spleen cells±S.E.	% Reduction	Mean HA titre±S.E.
		N. dubius†	SRBC					
A	4	−d28	+	7,950±741	78·9	5·29±0·48	78·4	4·25±0·25
B	4	−d14	+	10,050±1,861	73·4	5·78±1·11	76·4	5·25±0·25
C	4	d0	+	11,550±834	69·4	7·86±1·09	67·9	5·75±0·48
D	4	−	+	37,750±3,402	—	24·50±3·96	—	6·75±0·25
E	2	—	—	2,200±200	—	1·21±0·21	—	<3

*male CFLP mice.

†The pooled mean worm recovery from all the infected groups was 327·7±24·9.

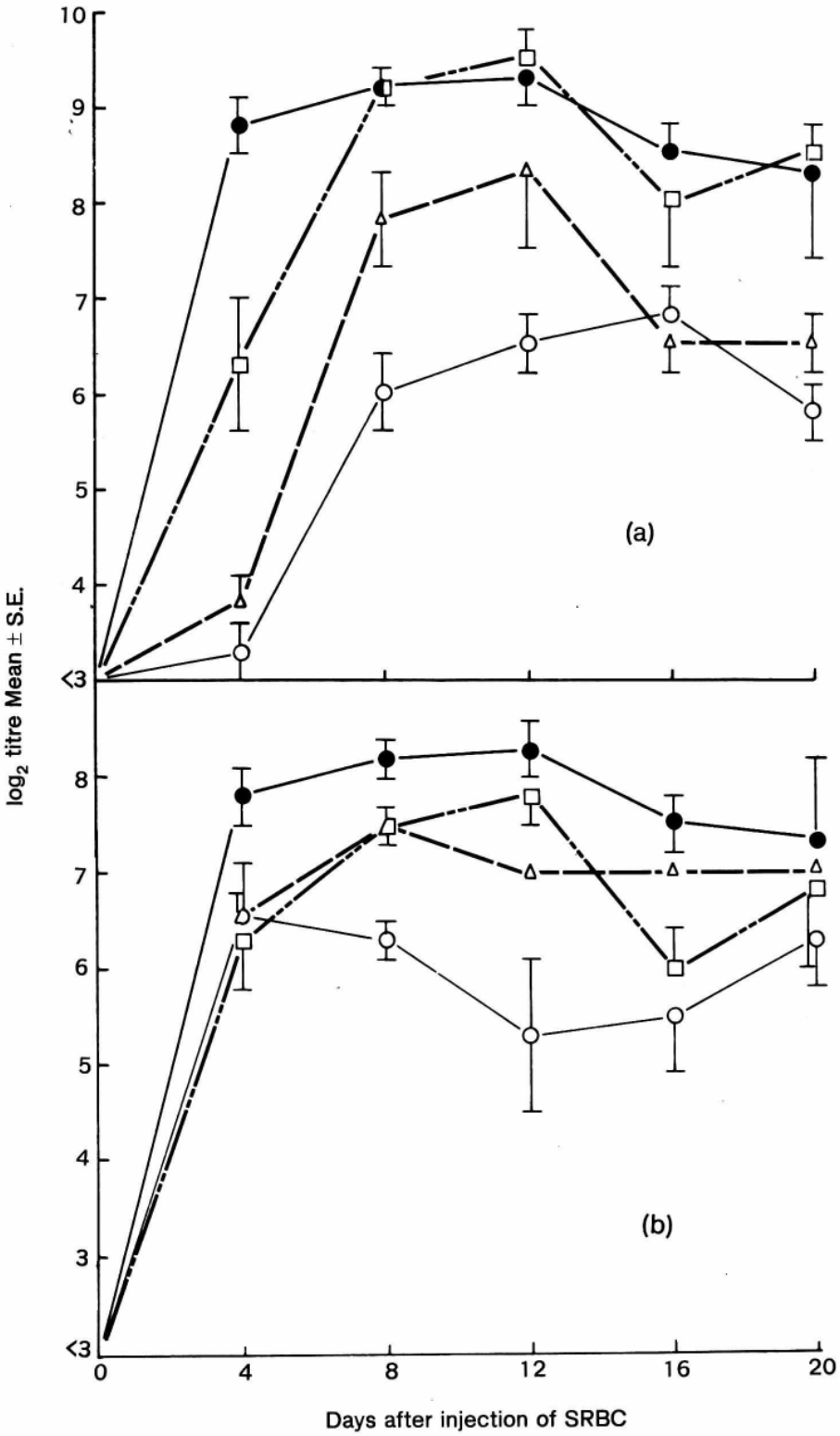

FIG. 1. The HA antibody response on different days after the administration of SRBC to CFLP mice infected with 500 (Fig. 1 a) or 50 (Fig. 1 b) *N. dubius* for various periods of time. The key to the symbols used is as follows: Control group—no infection, —●—; group given *N. dubius* on d0, —□ ○ group given *N. dubius* on d−14, —○—; group given *N. dubius* on d−28, —△—.

to SRBC (Table I). Over-all, the most severe depression of HA response was found in mice infected for 14 days before the administration of SRBC, but even mice infected on the day of SRBC injection had significantly depressed responses and a 69% reduction in direct PFC/spleen (Table II). However, Fig. 1 shows that in mice given *N. dubius* and SRBC on the same day, the HA titre rose by day 8 to a level which was indistinguishable from that in control mice. Therefore in these animals the initial phase of the response was slower, but the peak and declining phase titres were unaffected.

Number of N. dubius *required to affect the response to SRBC*

Further experiments were carried out to measure the HA response to SRBC in mice infected with different numbers of *N. dubius*. The results of two representative experiments are shown in Table III and Fig. 2.

Table III presents the results of an experiment in which the direct PFC to SRBC were measured in groups of four CFLP mice four days after injection of SRBC. The experimental groups (A, B, C) were given 500, 250 or 50 larvae of *N. dubius* 14 days earlier. It is quite apparent from these results that all three groups of infected mice had severely impaired responses to SRBC, ranging from a 50·1% to 87·8% reduction in PFC/spleen.

The full time course of the HA response to SRBC in mice infected with 500, 250 or 50 larvae of *N. dubius* is shown in Fig. 2. The data shows that on day 4 there was a clear dose related effect on the HA titre but thereafter all the infected mice had significantly reduced peak and declining phase HA titres. Additional experiments (results not shown) confirmed that within the range 50 to 500 larvae of *N. dubius*, all infection levels caused a depression of the HA response to concurrently administered SRBC. Immunodepression was less marked, however, in mice given longer infections, especially when the animals were infected with 50 larvae.

The effect of the route of antigen presentation

The route by which antigen is presented to an animal may influence the type of responses elicited as well as the degree to which the host response may be depressed in infected animals. Therefore, in this section we describe the results of an experiment in which we compared the response to SRBC in control and infected mice challenged either by i.p. or i.v. injection. Fig. 3 summarizes the results of an experiment in which groups of CFLP mice were infected with 150 or 400 larvae of *N. dubius* on day 14 and were challenged with 5×10^7 SRBC on day nought. This experiment also included groups of mice infected with 50 *N. dubius* but because the results were similar for all the infected groups, the data are not shown. It can be seen that the HA response in control mice was almost identical irrespective of the route by which antigen was injected. Likewise, in mice infected with *N. dubius*, the extent and general pattern of the depressed response was similar whether SRBC were given by i.p. or i.v. injection.

This experiment was repeated several times and it was confirmed by both HA titres and the PFC response that mice infected with *N. dubius* respond similarly to i.p. or i.v. injected SRBC.

Determination of 2ME sensitive and resistant HA response in mice infected with N. dubius

Ten CFLP mice were arranged into two groups of five animals. One group was infected with 400 larvae of *N. dubius* on day 14 and both groups were injected i.p. with 5×10^7 SRBC on day nought. The concentration of 2ME sensitive and resistant HA was determined on days after the administration of SRBC and the results are shown in

TABLE III. Direct PFC to SRBC in mice infected with different nos of *N. dubius*

Group	No.* mice	Treatment		Mean PFC/spleen ±S.E.	% Reduction	Mean PFC/10^6 spleen cells ±S.E.	% Reduction
		N. dubius†	SRBC				
A	4	500	+	5,500±265	87·8	1·99±0·29	90·7
B	4	250	+	12,700±2,511	71·7	6·28±2·29	70·7
C	4	50	+	22,400±3,398	50·1	5·84±1·18	72·7
D	4	—	+	44,900±3,991	—	21·40±4·9	—
E	2	—	−	1,800±800	—	0·64±0·06	—

*male CFLP mice.
†The MWR were as follows: Group A, 524·0±6·4; Group B, 207·3±12·3; Group C, 55·3±6·7.

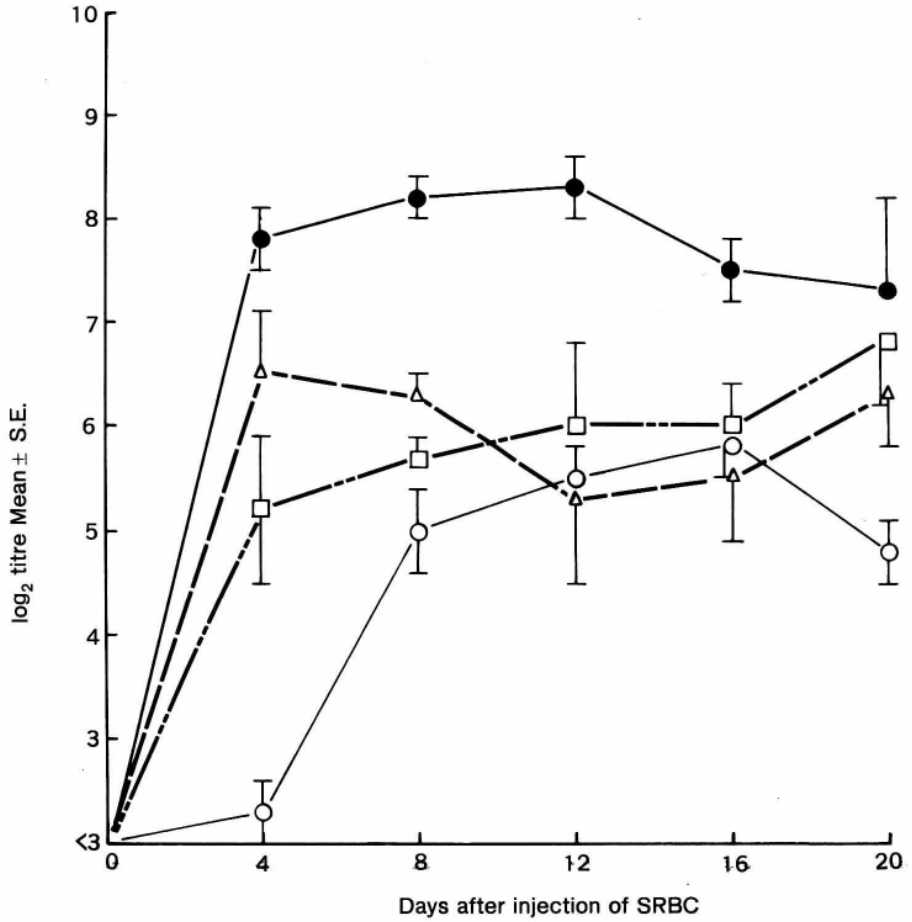

FIG. 2. The HA antibody response on different days after the administration of SRBC to NIH mice given different nos. of *N. dubius* larvae. The key to the symbols used is as follows: Control group—no infection, —●—; group given 500 *N. dubius*, —○— (MWR=463·3±7·2); group given 250 *N. dubius*, —□— (MWR=272·8±8·7); group given 50 *N. dubius*, —△— (MWR=66·8±5·5).

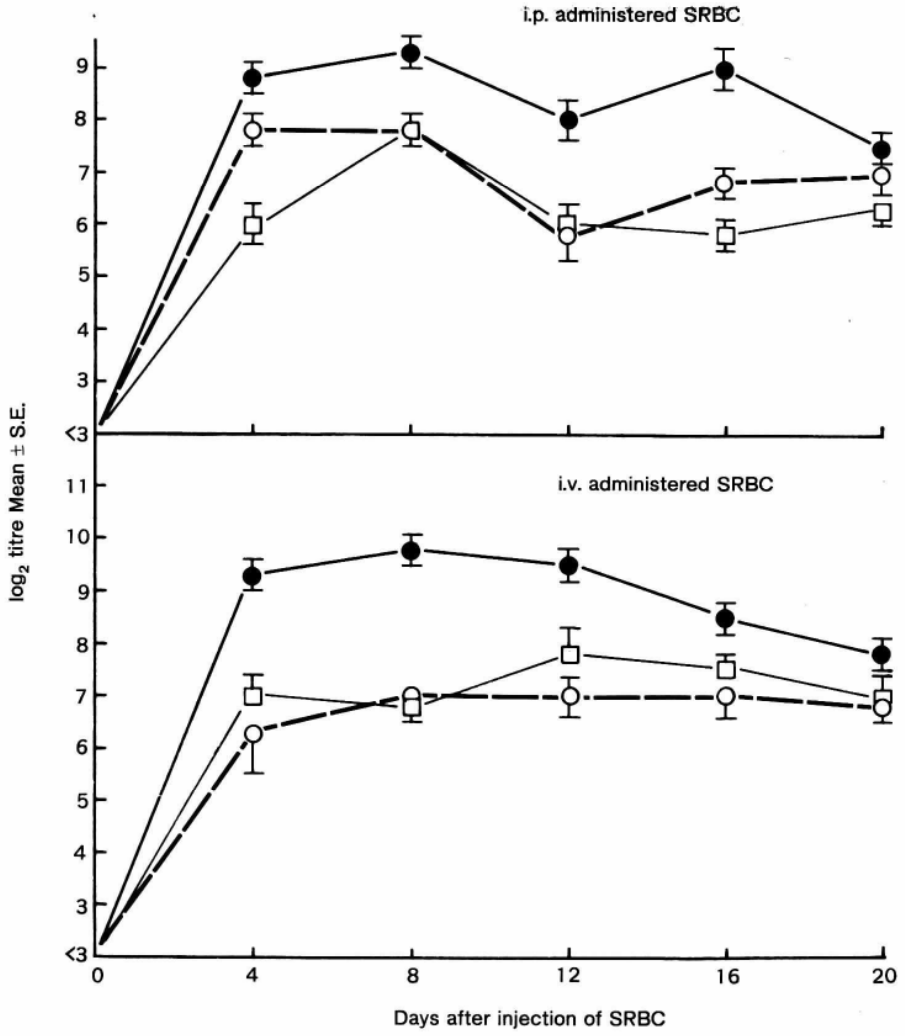

FIG. 3. The influence of the route of antigen presentation on the HA antibody response to SRBC of CFLP mice infected with *N. dubius*. The experimental groups were given 400 (—□—) or 150 (—○—) larvae of *N. dubius* on d − 14 and were challenged together with a control group (—○—) with SRBC by either i.p. or i.v. injection.

TABLE IV. The 2ME sensitive and resistant HA response in CFLP mice infected with *N. dubius*

	Control group		Group infected with *N. dubius*	
Day	−2ME	+2ME	−2ME	+2ME
4	9·0±0·3	<3	6·0±0·3	<3
8	10·6±0·2	4·4±0·4	8·0±0·3	3·6±0·5
12	10·4±0·2	9·8±0·2	7·6±0·7	5·4±0·2
16	8·4±0·2	8·0±0·3	8·0±0·3	7·0±0·3
20	7·0±0·3	6·8±0·4	7·2±0·4	5·8±0·6

*MWR = 454·4±7·5.

Table IV. It can be seen that treatment with 2ME completely abolished the HA titre on day 4 confirming that in both groups the HA was IgM. Similarly, there was a severe reduction in the titre of both groups after treatment with 2ME on day 8 but by day 12 the HA in control mouse sera was completely resistant to 2ME, whereas that in the sera from infected mice remained partially sensitive.

DISCUSSION

The chronic survival of nematode parasites is an intriguing problem, whose resolution will have profound implications for human and veterinary medicine. Although a number of hypotheses have been proposed to explain how nematodes might survive in otherwise fully immunocompetent hosts, none of these are supported by convincing evidence of a functional significance for the parasites concerned. In common with some species of protozoa, parasitic helminths may cause non-specific immunodepression in the host (HAIG *et al.*, 1980; FAUBERT & TANNER, 1971; ARAUJO *et al.*, 1977; PORTARO *et al.*, 1976), the precise significance of which is not understood, although it has been suggested that this may be a mechanism by which the organisms facilitate their own survival (OGILVIE & WILSON, 1976).

A particular problem in this respect is the observation that not only parasites which cause chronic infections, but also those which are effectively resisted by the host, may interfere with the host response to concurrently administered heterologous antigens (FAUBERT & TANNER, 1971; FAUBERT, 1977; CRANDALL & CRANDALL, 1976; HAIG *et al.*, 1980). Furthermore there is evidence that parasite-induced immunodepression does not affect the host response to parasite antigens. For example, in mice infected with *Trypanosoma brucei* the resultant immunodepression does not impair the immune response to trypanosome antigens (MACASKILL *et al.*, 1981). *N. dubius* may be an exception to this since there is now significant evidence that the presence of adult worms in the host intestine prevents the mouse from expressing an effective immune response to a challenge infection (HANNAH *et al.* 1982). The relationship of the depressed immune response to homologous and heterologous antigens in infected mice is an important point for clarification because both events may reflect the same phenomenon, namely, the parasite's mechanism for chronic survival.

In the experiments we reported here we have studied some of the factors which influence the response of mice infected with *N. dubius* to SRBC. It is evident that the greatest depression of the response to SRBC was associated with the higher levels of infection and with an interval of 14 days between infection and the administration of antigen. In these mice the HA titre was severely depressed, sometimes no antibodies at all being found on day 4. Eventually, most mice responded, although the peak titre was never as high as in control mice and during the subsequent decay period, less antibody was detected in their sera (Fig. 1). The importance of the relative timing of infection and injection of SRBC argues for a particular stage in the development of the parasite contributing significantly to immunodepression during this critical period. The larvae of *N. dubius* return to the gut lumen eight to nine days after infection, although in heavy infections there may be some retarded development and larval emergence may occur throughout the following week. This event is associated with intestinal changes which include inflammation and damage to the mucosa (BAKER, 1954; JONES & RUBIN, 1974). The damaged mucosa, loss of appetite and presumably parasite secretory/excretory products may combine to cause a traumatic event during which the weakened host is incapable of responding normally to administered antigens.

However, some experiments indicated that mice infected with 50 larvae of *N. dubius* were also incapable of mounting a normal response to SRBC (Fig. 1, Table III). At this

level of infection the parasite does not cause extensive pathogenesis. Mice can tolerate far heavier worm burdens for eight months or so (EHRENFORD, 1954; BEHNKE & HANNAH, 1982). Depressed responses were also observed in mice which had been infected for four or five weeks, by which time intestinal changes might have been expected to have stabilized. It is, therefore, conceivable that the immunodepression seen in low-level and/or long infections, reflects a generalized non-specific depression in the host's immune system which stems from the mechanism used by the parasite to evade host immunity.

The most marked effect of *N. dubius* was on the early, presumably IgM mediated, response. Thus direct PFC/spleen were reduced by 50% in mice infected with 50 larvae for 14 days. During a primary infection with *N. dubius* IgG_1 levels increase in the serum and stabilize at approximately double the control level within two to three weeks of infection (CRANDALL *et al.*, 1974; WILLIAM & BEHNKE, 1983). Equally, there is an increased catabolism of IgG, a finding which led BROWN *et al.* (1976) to propose that the increased catabolic activity and hence the shortened half-life of IgG in infected mice, may contribute to immunodepression. However, over-all IgM levels are hardly affected by infection with *N. dubius* (see MOLINARI *et al.*, 1978; PROWSE *et al.*, 1978; WILLIAMS & BEHNKE, 1983) and, therefore, elevated catabolism of serum immunoglobulins is unlikely to be a satisfactory explanation of our results. This conclusion is also supported by the results in Table I which show that *N. dubius* larvae given four or eight days after SRBC did not depress the HA response in the following period. Whilst infection with *N. dubius* would have been expected to increase the IgG catabolic rate in these mice, it had no detectable effect on the concentration of antibody to SRBC, which would have been of both IgM and IgG isotype.

It was important in this study to determine whether the observed immunodepression in infected animals was dependent on the route by which antigen was presented. MITCHELL & HANDMAN (1977) found that reduced immune responses in mice infected with *Mesocestoides corti* were demonstrable when antigen was presented i.p. but not when mice were injected i.v. The authors suggested that sequestration of antigen, and its subsequent local destruction, accounted for the markedly suppressed systemic responses induced by i.p. injected antigens. Clearly, such an explanation is not relevant to our study, since there was a remarkable similarity between the extent and pattern of the depressed responses to i.v. and i.p. injected SRBC in mice infected with *N. dubius*.

It is important to emphasize here that the development of *N. dubius* in its host falls into two quite distinct phases. The larval L3 and L4 develop in the intestinal walls whereas the adults live in the gut lumen. Therefore, there may be two qualitatively distinct stage-specific processes involved in evading host responses appropriate for the elimination of each phase. Developing larvae might need only to slow down the hosts' response sufficiently to enable the completion of their development and escape into the intestinal lumen. In immune animals the larval stages of *N. dubius* are destroyed by a granulomatous response (JONES & RUBIN, 1974; BEHNKE & PARISH, 1979) which would be of little value against adult worms in the gut lumen. The rejection of mature *N. dubius* is a controversial subject. There is some evidence that adult parasites can be expelled in particular circumstances (BEHNKE & WAKELIN, 1977; HURLEY *et al.*, 1980) but expulsion, when it does take place, occurs slowly over a period of a week or more and is not the sudden event that characterizes the expulsion of *T. spiralis* and *Nippostrongylus brasiliensis*. The difficulty which the mouse experiences in rejecting *Nematospiroides dubius* could be explained by a mechanism involving the parasite preventing the host from expressing an effective immune response throughout the eight-month period of a primary infection.

An attractive hypothesis and one which is being investigated at present, is that secretory factors from the parasite initiate non-specific as well as specific suppressor cell activity. Our experiments suggest that it may be more relevant to study long-standing infections, rather than the early phase of infection when additional toxic and pathogenic consequences may contribute to over-all immunodepression. It would obviously be of little value to the parasite to depress its host severely but a generalized weakening of host immunocompetence may be an inevitable consequence of a mechanism which operated more specifically to suppress the expression of homologous immunity at the intestinal level.

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