Pergamon

RESISTANCE OF THE HOOKWORMS *ANCYLOSTOMA CEYLANICUM* AND *NECATOR AMERICANUS* TO INTESTINAL INFLAMMATORY RESPONSES INDUCED BY HETEROLOGOUS INFECTION

JERZY M. BEHNKE,* RICHARD ROSE and JENNIFER LITTLE

MRC Experimental Parasitology Research Group, Department of Life Science, University of Nottingham, University Park, Nottingham NG7 2RD, U.K.

Abstract—BEHNKE J. M., ROSE R. and LITTLE J. 1994. Resistance of the hookworms *Ancylostoma ceylanicum* and *Necator americanus* to intestinal inflammatory responses induced by heterologous infection. *International Journal for Parasitology* **24**: 91–101. Experiments were carried out to ascertain whether the acute inflammatory phase of the intestinal response of hamsters to infection with *Trichinella spiralis* would adversely affect hookworms in concurrently infected animals. The survival and growth of hookworms were unaffected. However, the presence of hookworms reduced the establishment of *T. spiralis*, the initial growth of female worms and their fecundity. The expulsion of *T. spiralis* was also significantly slower in concurrently infected animals and there was significant depression of the serum IgG antibody response to muscle stage and adult worm antigens of *T. spiralis* in concurrently infected animals. These results are discussed in relation to the chronicity of human hookworm infections.

INTRODUCTION

HUMAN hookworms cause persistent chronic infections both after experimentally administered single pulse infections (Beaver, 1988) and during natural exposure in the field (Bundy, 1990). Moreover, there is little evidence from field studies to support a major role for acquired immunity in regulating human hookworms in communities continuously exposed to infection (Behnke, 1987a, 1991a), although there is good evidence that related hookworm species affecting canine hosts are controlled by host immune responses (Miller, 1971). The reasons for the chronicity of human hookworm infections are not understood but the possibility exists that human hookworms are resilient in the face of host effectors and cannot be eliminated by the intestinal responses generated in the human host.

Following specific activation of CD4+ lymphocytes by antigens liberated from nematodes resident in the intestine, the secretion of cytokines associated with Th-2 cells results in local infiltration of the mucosa by inflammatory cells and eventually in the generation of an environment in which worms cannot survive. The final effector mechanism is still not understood (Moqbel & MacDonald, 1990), but the inflammatory phase of the intestinal response is known to act non-specifically since unrelated parasites, concurrently resident in the intestine are also rejected (Kennedy 1980; Behnke, Cabaj & Wakelin, 1992; Behnke, Bland & Wakelin, 1977; Christie, Wakelin & Wilson, 1979; Ferretti, Gabriele, Palmas & Wakelin, 1984) or have their growth severely impaired (Howard, Christie, Wakelin, Wilson & Behnke, 1978).

In this paper we report on experiments in which we investigated the ability of the hookworms *Necator americanus* and *Ancylostoma ceylanicum* both of which cause patent infections in man (Behnke, 1990), to withstand the consequences of intestinal inflammation heterologously induced by *Trichinella spiralis* (see Rothwell, 1989). Our experiments were carried out in hamsters, which are susceptible to both species of hookworms and to *T. spiralis*, in order to determine whether hookworms would be expelled during the acute inflammatory phase of the response to *T. spiralis* or would survive a response which has severe consequences for other intestinal parasites.

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*To whom all correspondence should be addressed.

MATERIALS AND METHODS

Parasites and hosts. Infective larvae of *N. americanus* were obtained in 1983 from Dr Rajasekariah of CIBA-GEIGY Hindustan Ltd, Bombay, India and the parasite has been maintained since by regular passage through hamsters as described originally by Sen (1972) and Behnke, Paul & Rajasekariah, 1986). It is important to note here that *N. americanus* will mature to patency only following exposure of neonatal (1–3 day old) hamsters to percutaneous infection (Rajasekariah, Deb, Dhage & Base, 1985). Experiments with *N. americanus* were therefore limited by the need for synchronized mating of female hamsters and by resulting litter sizes. *A. ceylanicum* was also obtained from Dr Rajasekariah and was passaged through adult hamsters using techniques which have been described by Garside & Behnke (1989). Larvae were administered orally to mature hamsters throughout this study. The strain of *T. spiralis* and the methods used for the infection of animals and recovery of worms have been described previously (Wakelin & Lloyd, 1976). Briefly, the small intestines were opened longitudinally and incubated for 6 h at 37°C, suspended in nylon mesh in beakers of Hanks's saline, in a water bath. After this time the mesh was removed carefully and the contents either examined immediately or fixed by the addition of concentrated formol ethanol. Both the tissues contained in the mesh and the mesh were carefully examined for the presence of remaining hookworms and pieces which may have been cut at autopsy but in general the majority of worms migrated into the saline and were counted subsequently in Petri dishes under the low magnification of a binocular microscope. Assays of female worm fecundity were carried out as described by Wakelin & Wilson (1977). All the animals used in this work were syngeneic DSN hamsters originally purchased from Intersimian Ltd, Oxford, U.K., but now maintained, under conventional conditions with access to food and water *ad libitum*, as a closed breeding colony in the Department of Life Science at Nottingham University. Both sexes were used but, with the exception of experiment 5 involving *N. americanus*, the animals comprising any single experiment were of the same sex, as stated. Neither the establishment nor the course of infection with *N. americanus* and *A. ceylanicum* in hamsters differ between the sexes (Rose & Behnke, 1990; Garside & Behnke, 1989). Experimental groups were set up in separate cages 1–2 weeks before infection.

Preparation of antigens. Outbred CFLP mice were infected orally with *T. spiralis* and L1 antigen was obtained from muscle digests as described by Wakelin & Lloyd (1976). Adult worm homogenate was prepared from worms collected 7 days p.i. The parasites were washed 10 times in ice-cold sterile PBS and were homogenized in minimal volume of PBS using a glass tissue homogenizer held in an ice bath. The resulting suspension was centrifuged at 10,000 g for 1h at 4°C to remove coarse particulate matter. The supernatant was filtered (0.22 µm filter, Millipore), analysed for protein concentration using a method modified from Lowry, Rosebrough, Farr & Randall (1951), aliquoted and stored at −40°C.

Measurement of antibody responses. Parasite specific IgG responses were measured by a standard ELISA. Briefly, ELISA microtitre plates were coated with 50µl of worm antigen (5 mg ml⁻¹). Alkaline phosphatase conjugated Protein A (sigma) was used to measure specific responses. After addition of the substrate (P-nitrophenylphosphatase) colour changes were read at 410 nm on a Dynatech MR700 Microplate Reader. Sera were assayed individually in triplicate at a dilution of 1 in 1000, after storage at −40°C.

Measurement of worms. All the worms recovered at autopsy were collected, fixed in formol 70% ethanol (37°C) and were kept in separate tubes until measured. They were drawn to scale using a camera lucida arrangement attached to a microscope. The drawings were then measured using a digitizer pad and an IBM computer with a program for conversion of lengths traced into discrete units (courtesy of Dr R. Ramsey). Ten worms of both sexes were measured from each hamster and a mean value for the length of male and female worms from individual hamsters was derived. These mean values were then used to calculate the overall group mean.

Mast cell counts. A 2 cm length of small intestine taken 10 cm from the pyloric sphincter was fixed in Carnoy's fixative and processed using standard histological techniques. Sections cut at 5 µm were stained with Alcian Blue, counter-stained with Safranin O and mounted in DPX using the method of Alizadeh & Wakelin (1982) with the following modifications. Sections were stained for 25–30 minutes in Mayer's haematoxylin, then for 20–25 minutes in phosphate buffered Safranin O before processing and mounting in DPX. Five non-adjacent sections were examined and in each the mast cells evident in 20 villus crypt units (VCU) were counted. Totals counted on each section were averaged to yield the number of mast cells/20 VCUs for each animal and these values were used to generate a mean value/20VCUs for pertinent experimental groups.

Statistical analysis of results. For clarity data are presented as group mean values ± S.E.M. Non-parametric statistical procedures were used to analyse the data sets, because normal distribution of data could not be assumed. When a difference was expected in a specific direction, *a priori* hypotheses were tested by the procedures described by Meddis (1984) and the test statistic z is given as appropriate. In the event of a hypothesis being rejected [P = Not significant (NS)], groups were compared by a general one-way ANOVA in order to determine whether there were any significant differences between the groups and the statistic H as given. A two-way ANOVA (Meddis, 1984) was employed to assess the effects on growth of worms, specifying infection (concurrent vs single) and worm sex (male vs female) as the two factors. Relationships over time within experimental groups were examined by the Spearman Rank Order Correlation Test and the statistic r_s is given. Probabilities (P) of 0.05 or less were considered significant but throughout exact probabilities are given. In certain cases, multiple analyses could not be avoided, and, where these were undertaken, the cut-off value for significance was lowered to

$P = 0.025$ or 0.01 (depending on No. of tests) in order to avoid Type I errors.

EXPERIMENTAL DESIGN AND RESULTS

Establishment of Trichinella spiralis *in hamsters carrying a patent infection with* A. ceylanicum *(exp 1)*

It was necessary to determine first whether the establishment of *T. spiralis* would be affected by the presence of adult *A. ceylanicum*. Twenty-three male hamsters were arranged into three groups. Groups A ($n = 8$) and C ($n = 5$) were infected with 60 L3 of *A. ceylanicum* on day -14. Group A and group B ($n = 10$) were then challenged with 500 muscle larvae of *T. spiralis* on day 0. All the groups were killed for worm recovery 7 days later and the results are presented in Table 1. The fecundity of female *T. spiralis* was assayed in 5 hamsters from groups A and B on the day of autopsy and a sample of worms for each hamster was measured for length.

The results of worm recoveries were analyzed by a general test since we had no *a priori* grounds for expecting a difference in worm counts in either direction. The establishment of *T. spiralis* was significantly impaired in hamsters carrying a patent hookworm infection (group A, 18.8% reduction relative to the control group B, $H = 6.2$, n_1, $n_2 = 8$, 10, $P = 0.013$) as measured by worm recovery 7 days p.i. We then tested the specific hypothesis that the growth of *T. spiralis* would be slower in concurrently infected animals by a two way ANOVA, but the analysis gave $z = 1.27$, $P = NS$. Nevertheless, comparison of mean values showed that male and female worms for concurrently infected animals were 91.6 and 90.6%, respectively of the length of those from hamsters carrying *T. spiralis* alone. Subsequent separate analysis of the sexes from concurrently infected and control animals by a general test gave $H = 6.88$, n_1, $n_2 = 8$, 10, $P = 0.009$ for female worms and $H = 0.87$, n_1, $n_2 = 8$, 10, $P = NS$ for males. There was no significant effect of concurrent infection on the fecundity of female worms (general test $H = 1.32$, n_1, $n_2 = 5$, 5, $P = NS$), although fecundity was lower and more variable than in the control group with one of the five hamsters harbouring females yielding <2 larvae/female/h and another >5 larvae/female/h.

Establishment and course of infection with Trichinella spiralis *in hamsters carrying mature* A. ceylanicum *(exp 2)*

The second experiment was carried out to confirm the results of exp 1 and to determine whether the presence of *A. ceylanicum* would alter the subsequent course of infection with *T. spiralis* in hamsters. Twenty-five female hamsters, arranged in 5 groups

[$n = 7$ (A), 6 (B), 6 (C), 3 (G) and 3 (I)] were infected with 50 L3 of *A. ceylanicum* on day -15. On day 0 groups A, B and C were challenged with 500 muscle larvae of *T. spiralis*. A further 21 sex- and age-matched hamsters, arranged in three groups of 7 each (D, E and F), were also infected with the same dose of *T. spiralis* on day 0. Two additional groups (H and J, $n = 3$ in each case) remained uninfected with either parasite throughout in order to provide sera from naive animals for antibody assays. The results are summarized in Figs. 1 and 2 and Table 2.

Following the results of exp 1, the *a priori* hypothesis that the presence of hookworms would impair the establishment of *T. spiralis* was examined in a specific test. The establishment of *T. spiralis* in hamsters carrying a patent *A. ceylanicum* infection (group A), as assessed by worm counts on day 7 p.i., was significantly reduced (20.6% reduction relative to control group (D), $z = 1.86$, n_1, $n_2 = 7,7$, $P = 0.0314$). Two weeks p.i., *T. spiralis* worm burdens were similar in single and concurrently infected animals (general test groups B vs E, $H = 1.31$, n_1, $n_2 = 6,7$, $P = NS$) but by day 21, the concurrently infected hamsters (group C) had more *T. spiralis* than those given *T. spiralis* alone (group F; $H = 5.9$, n_1, $n_2 = 6,7$, $P = 0.015$). As in exp 1 there was no overall significant effect of concurrent vs single infection on the size of worms on day 7 (specific prediction by two-way ANOVA, $z = 1.61$, $P = NS$) but comparison of mean worm lengths showed that male and female worms from the concurrently infected animals (group A) were 90.1 and 71.6% respectively of the length of those from the control group (D). Analysis by a general test again indicated that female but not male worms from concurrently infected animals were smaller ($H = 9.843$, n_1, $n_2 = 7,7$, $P = 0.002$; $H = 1.894$, n_1, $n_2 = 7,7$, $P = NS$) relative to those of the control group (D). By day 14 the mean length of female worms from concurrently infected hamsters (group B) was 96.3% of that from controls (group E). However, male worms from group B were smaller than those from group E (92.9% of the length of group E males) and the difference was just significant (general test $H = 4.067$, n_1, $n_2 = 6,7$, $P = 0.044$). The fecundity of female worms from 4 hamsters in the concurrently infected and *T. spiralis* only groups was measured on day 7. The mean value for the concurrently infected group was 1.69 ± 0.11 larvae/female/h whilst that for worms from the control group was 3.55 ± 0.38 (Specific test, $z = 2.31$, n_1, $n_2 = 4,4$, $P = 0.0104$).

In contrast *A. ceylanicum* were not affected by the host response to *T. spiralis*. There was no significant loss of worms from concurrently infected hamsters during the course of the experiment ($r_s = -0.089$, $n = 19$, $P = NS$) and no difference in worm burdens

Table 1—Establishment of *Trichinella spiralis* in hamsters carrying a patent infection with *Ancylostoma ceylanicum* (Experiment 1)

Group	No. of hamsters	Mean no worms recovered ± S.E.M.	*T. spiralis** Mean length ± S.E.M. (mm) Females	Males	Fecundity‡ L1/F/h	*A. ceylanicum*† Mean No. worms recovered ± S.E.M.
(A) *A. ceylanicum* + *T. spiralis*	8	228.3 ± 11.8§	2.88 ± 0.1	1.30 ± 0.11	3.76 ± 0.44	22.4 ± 4.7
(B) *T. spiralis* alone	10	281.1 ± 12.7§	3.18 ± 0.29	1.43 ± 0.22	4.15 ± 0.08	—
(C) *A. ceylanicum* alone	5	—	—	—	—	29.6 ± 6.6

* 500 larvae of *T. spiralis* were given on day 0 and all the animals were killed for worm counts on day 7.
† 60 larvae of *A. ceylanicum* were administered on day −14, i.e. 2 weeks before challenge with *T. spiralis*.
‡ Fecundity is expressed as mean no. of larvae/female worm/h of incubation.
Statistical analysis of results: § $H = 6.2$ $P = 0.013$. For analysis of other groups see text.
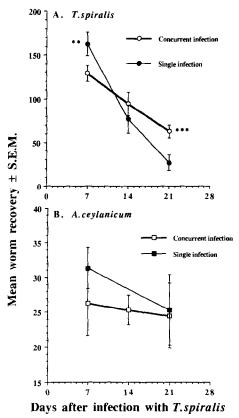
FIG. 1. Recovery of *T. spiralis* and *A. ceylanicum* from hamsters with concurrent or single species infections (Exp 2). Statistical analysis of results: ** $z = 1.86$, $P = 0.0314$, *** $H = 5.9$, $P = 0.015$.
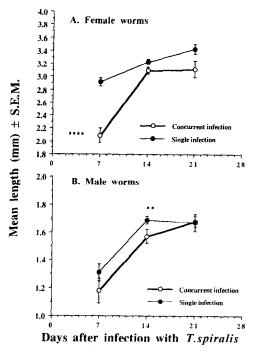

FIG. 2. The length of *T. spiralis* recovered from hamsters infected only with *T. spiralis* or concurrently infected with *A. ceylanicum* (Exp 2). Statistical analysis of results: **** $H = 9.843$, $P = 0.002$, ** $H = 4.067$, $P = 0.044$.

between concurrently infected animals and hamsters carrying *A. ceylanicum* alone on either day 7 or day 21 p.i.

Antibody responses to L1 and adult *T. spiralis* antigens were measured on day 21 p.i. with *T. spiralis* and the results are presented in Table 2. Sera taken earlier than day 21 did not show reactivity against these antigens. On day 21 p.i. all the hamsters infected with *T. spiralis* (groups C and F) responded against both antigens relative to uninfected controls. The response against adult worm antigens was significantly greater in hamsters with *T. spiralis* alone (specific test for group C vs F, $z = 1.71$, $P = 0.044$) but there was no significant difference in the response to larval antigen ($z = 0.43$, $P = $ NS).

*Survival of an established infection with
A ceylanicum during the acute phase of the response
to Trichinella spiralis (exps 3 and 4)*

The ability of *A. ceylanicum* to survive the inflammatory response to *T. spiralis* was examined further in exp 3 and 4. Both experiments were carried

out employing male hamsters and the experimental design was similar except that the interval between infection with *A. ceylanicum* and challenge with *T. spiralis* was increased relative to exp 2 to 3 weeks (exp 3) and 4 weeks (exp 4). The results are summarized in Table 3. There was no loss of *A. ceylanicum* despite the loss of 61.7% (exp 3) and 90.9% (exp 4) of *T. spiralis* during the 3 week (exp 4) and 4 week (exp 3) periods following challenge with *T. spiralis*. Moreover, there was no indication in either experiment that the establishment of *T. spiralis* was impaired in concurrently infected animals.

*Survival of an established infection with
N. americanus during the acute phase of the host
response to Trichinella spiralis (exp 5)*

The experimental design employed in expts 3 and 4 was used to determine whether *N. americanus* was equally resistant to the non-specific effects of the inflammatory response elicited by *T. spiralis*. Litters of neonatal hamsters, including both sexes, were exposed percutaneously to 140 infective larvae of *N. americanus* when 2 days old and were challenged with *T. spiralis* 5 weeks later. One group of hamsters ($n = 4$) was killed on the day of challenge, i.e. 5 weeks after exposure to *N. americanus*, to confirm establishment

TABLE 2.—ANTIBODY RESPONSES TO MUSCLE STAGE LARVAL (L1) AND ADULT WORM HOMOGENATE ANTIGENS OF *Trichinella spiralis* IN HAMSTERS CONCURRENTLY INFECTED WITH *Ancylostoma ceylanicum* (EXPERIMENTS 2 AND 6).

Group*	Mean optical density ± S.E.M.								
	Exp 2			Exp 6					
	A. ceylanicum given day −15. Sera from day 21 p.i.			*A. ceylanicum* given on day 8. Sera from day 28 p.i.			*A. ceylanicum* given on day 14. Sera from day 35 p.i.		
	No. of hamsters	Adult worm antigen	L1 antigen	No. of hamsters	Adult worm antigen	L1 antigen	No. of hamsters	Adult worm antigen	L1 antigen
A. ceylanicum +*T. spiralis*	6	0.238 ± 0.010†	0.266 ± 0.033	9	0.369 ± 0.021‡	0.472 ± 0.045§	9	0.422 ± 0.036‖	0.411 ± 0.061**
T. spiralis alone	7	0.278 ± 0.015†	0.264 ± 0.012	4	0.866 ± 0.380‡	0.763 ± 0.109§	4	0.582 ± 0.018‖	0.687 ± 0.069**
A. ceylanicum alone	3	0.184 ± 0.006	Nd	7	0.020 ± 0.006	0.115 ± 0.002	7	0.050 ± 0.004	0.144 ± 0.007
Uninfected	3	0.171 ± 0.006	0.145 ± 0.002	–	Nd	Nd	–	Nd	Nd

* For details see text. Nd = Not measured.

Statistical analysis: Groups with the same symbols have the following statistical values.

† $z = 1.71$, $P = 0.0436$.

‡ $z = 2.47$, $P = 0.0068$.

§ $H = 4.67$, $P = 0.031$.

‖ $z = 2.62$, $P = 0.004$.

** $H = 5.36$, $P = 0.021$.

Table 3—Survival of *A. ceylanicum* during the acute phase of the response to *T. spiralis*

| Group | Mean no. of worms recovered ± S.E.M. on days after infection with *T. spiralis* [n] | | | | |
	A. ceylanicum			*T. spiralis*	
Experiment 3	0	5	21	5	21
A. ceylanicum only	28.3 ± 6.1 [5]	34.5 ± 2.8 [6]	23.2 ± 1.6 [6]	–	–
A. ceylanicum + *T. spiralis*	Nd	25.7 ± 4.9 [3]	28.5 ± 3.0 [6]	251.3 ± 10.1 [3]	96.2 ± 15.6 [6]
T. spiralis only	–	–	–	270.5 [2]	122.8 ± 13.8 [4]
Experiment 4	0	7	28	7	28
A. ceylanicum only	35.8 ± 9.4 [5]	Nd	22.8 ± 12.0 [5]	–	–
A. ceylanicum + *T. spiralis*	Nd	37.5 ± 6.6 [4]	37.0 ± 9.5 [4]	104 ± 2.0 [4]	9.5 ± 4.3 [4]
T. spiralis only	–	–	–	87.8 ± 10.4 [6]	43.4 ± 13.9 [5]

In Exp 3 hamsters were given 47 L3 of *A. ceylanicum* on day −21 and were challenged 3 weeks later with 480 muscle larvae of *T. spiralis*

In Exp 4 hamsters were given 95 L3 of *A. ceylanicum* on day −28 and were challenged 4 weeks later with 400 muscle larvae of *T. spiralis*

of worms and 23.8 ± 6.1 worms were recovered. A second group ($n = 5$), not challenged with *T. spiralis*, was killed 21 days later to determine the survival of *N. americanus* in the absence of *T. spiralis*, and 38.4 ± 11.3 worms were recovered. The difference in recovery of worms was not statistically significant. Seven hamsters carrying *N. americanus* were challenged with *T. spiralis* (490 muscle larvae) and were killed for worm counts either 5 ($n = 4$) or 21 ($n = 3$) days later. Eight uninfected age-matched hamsters were infected only with *T. spiralis* and were killed on the same days [Day 5 ($n = 4$) and 21 ($n = 4$)]. Figure 3 summarizes the results. There was no impairment of the establishment of *T. spiralis*, nor was the duration of infection with *T. spiralis* prolonged significantly by the presence of *N. americanus* in concurrently infected animals. Despite some variation in the hookworm burdens, the survival of *N. americanus* to day 21 after challenge with *T. spiralis* was not impaired in concurrently infected animals, despite the loss of 92.5% of *T. spiralis* over this interval.

Establishment of A. ceylanicum *during the acute phase of the host response to* T. spiralis *(Experiment 6)*

Finally an experiment was carried out to determine whether the larval stages of *A. ceylanicum* are more susceptible to heterologously elicited intestinal inflammation. It was therefore necessary to infect animals first with *T. spiralis* and then to administer challenge inocula with *A. ceylanicum* at times adjudged to coincide with the onset of the acute expulsion phase of
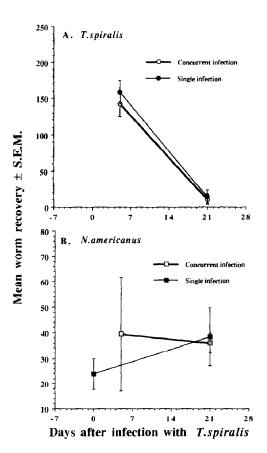

Fig. 3. Recovery of *T. spiralis* and *N. americanus* from hamsters with concurrent or single species infections (Exp 5).

T. spiralis. The experiment comprised 57 male hamsters which were divided into 10 groups (A–J). One group (J) of 4 hamsters was not infected, and provided sera from naive animals to control for antibody responses. One animal from this group was killed on each of days 8, 14, 21 and 35 relative to infection with *T. spiralis* in the other groups. Five groups of 4 hamsters each were infected with *T. spiralis* and were killed for worm counts 8 (group E), 14 (group F), 21 (group G), 28 (group H) and 35 (group I) days p.i. Samples of intestinal tissue were also processed for mast cell counts from these animals. Two of the remaining groups (B, $n = 9$ and D, $n = 9$) were infected with *T. spiralis* but subsequently challenged orally with 61 L3 of *A. ceylanicum* on days 8 (group B) or 14 (group D). Groups A ($n = 7$) and C ($n = 8$) were infected with *A. ceylanicum* on the same days on which groups B and D were given hookworms and acted as controls for establishment and survival of hookworms. Groups A and B were killed 28 days after infection with *T. spiralis* (day 20 following *A. ceylanicum*) and groups C and D on day 35 (day 21 following *A. ceylanicum*). Worm burdens are presented in Fig. 4, the effect on the growth of *A. ceylanicum* is shown in Table 4 and antibody responses are recorded in Table 2.

Eight days after infection with *T. spiralis* a mean of 147 worms were recovered representing 29.4% establishment (Fig. 4A). By day 21, 85% of the worms had been rejected with the greater loss occurring 14–21 days p.i. Loss of worms was accompanied by mastocytosis with mast cell counts increasing from 58.8 ± 13.9/20 VCU on day 8 p.i. to 143.5 ± 13.44 on day 21 and 154.0 ± 2.8/20 VCU on day 35. There was a highly significant positive correlation between mast cell density and time after infection ($r_s = 0.803$, $n = 12$, $P = 0.002$). Concurrently infected animals did not harbour more worms than hamsters infected only with *T. spiralis* on day 28 (group B, 8.2 ± 1.2, specific test $z = -1.55$, $P = $ NS) nor on day 35 (group D, 14.4 ± 3.9, specific test $z = 0.62$, $P = $ NS).
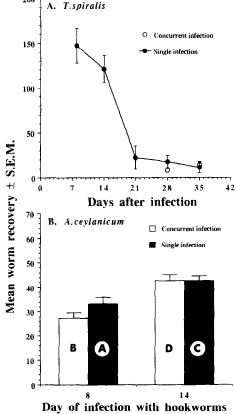
The establishment of *A. ceylanicum* was marginally different on the two occasions when larvae were given with 54% (group A) and 70% (group C) as assessed by worm recoveries from control hamsters on days 28 and 35 p.i. with *T. spiralis*, respectively. However, *A. ceylanicum* established and survived in the concurrently infected animals (groups B and D) without significant loss of worms relative to the control groups (groups A and C, specific test in both cases, $z = 1.43$ and 0.48, respectively, $P = $ NS). Moreover, there was little effect on the growth of *A. ceylanicum*: analysis by two-way ANOVA yielded $z = 1.31$ and $z = -0.25$ respectively, $P = $ NS. Direct comparison of worms of

FIG. 4. Recovery of *T. spiralis* and *A. ceylanicum* from hamsters with concurrent or single species infections (Exp 6). (A) The course of infection with *T. spiralis* in groups of 4 hamsters killed at times p.i. *T. spiralis* was also recovered on days 28 and 35 from two groups of hamsters challenged earlier (days 8 or 14 p.i.) with *A. ceylanicum*. (B) Recovery of *A. ceylanicum* from hamsters carrying single species or concurrent infections with *T. spiralis*. Hamsters were first infected with 500 muscle larvae of *T. spiralis* (groups B and D) and then either on days 8 (B) or 14 (D) were challenged, together with uninfected control groups (groups A and C, respectively), with 61 L3 of *A. ceylanicum*. The groups were killed for worm counts 20 (A and B) and 21 (C and D) days p.i., with hookworms, respectively.

each sex in turn by a general test did not detect significant differences.

Antibody responses of hamsters infected with *T. spiralis* and killed on days 28 (groups B and H) and 35 (groups D and I) were compared using *T. spiralis* adult worm and muscle stage (L1) antigens (Table 2). Following the results of Experiment 2 the specific test was applied to ELISA data derived employing adult worm antigen (group B vs H, $z = 2.47$ n_1, $n_2 = 9,4$, $P = 0.0068$; group D vs I, $z = 2.62$, n_1, $n_2 = 9,4$, $P = 0.0044$) indicating significant differences between concurrently and singly infected animals. The res-

TABLE 4—THE GROWTH OF *Ancylostoma ceylanicum* FOLLOWING ESTABLISHMENT DURING THE ACUTE PHASE OF THE HOST RESPONSE TO *Trichinella spiralis* (EXP 6)

Group	No. of hamsters	Mean length (mm) ± S.E.M.	
		Male worms	Female worms
A. ceylanicum given 8 days after *T. spiralis**			
(A) *A. ceylanicum* alone	7	5.06 ± 0.11	5.65 ± 0.11
(B) *A. ceylanicum* + *T. spiralis*	9	4.76 ± 0.10	5.49 ± 0.10
A. ceylanicum given 14 days after *T. spiralis*†			
(C) *A. ceylanicum* alone	8	5.08 ± 0.11	5.67 ± 0.09
(D) *A ceylanicum* + *T. spiralis*	8	5.14 ± 0.07	5.77 ± 0.11

* 500 larvae of *T. spiralis* were given to group B on day 0 and 61 larvae of *A. ceylanicum* to both groups on day 8. All the animals were killed for worm counts and measurements 20 days later on day 28.

† 500 larvae of *T. spiralis* were given to group D on day 0 and 61 larvae of *A. ceylanicum* to both groups on day 14. All the animals were killed for worm counts and measurements 21 days later on day 35.

See text for statistical analysis.

ponse to larval antigens was analyzed by a general test because expt. 2 had not indicated a significant difference in relation to this antigen but on this occasion there was a significant difference between groups B and H ($H = 4.67$, n_1, $n_2 = 9,4$, $P = 0.031$) and between D and I ($H = 5.36$, n_1, $n_2 = 9,4$, $P = 0.021$).

As an additional precaution to ensure that the concurrently infected hamsters had earlier harboured *T. spiralis*, the diaphragms of all hamsters were examined and it was confirmed that those in Groups B and D were heavily infected with L1 larvae.

DISCUSSION

The experiments described in this paper have shown conclusively that the hookworms *N. americanus* and *A. ceylanicum* have the capacity to survive the changes which occur in the intestinal environment during the acute phase of the host response to *T. spiralis* and, in this respect, they contrast with other species of GI nematodes and tapeworms which are severely affected when present during such responses (Behnke *et al.*, 1992). Hamsters infected with *T. spiralis* mounted a primary response the kinetics of which were similar to those described for mice and rats, with worm expulsion commencing after the first week of infection and > 90% loss of worms by week 4, accompanied by mastocytosis as in other rodents (Wakelin & Lloyd, 1976; Alizadeh & Wakelin, 1982). Our results indicate that hookworms can withstand a temporary phase of heterologously-induced inflammation in the intestine of their hosts and suggest that they are resilient in the face of host-immune effectors, possibly explaining why human hookworms are chronic under field conditions.

One explanation for our data may be that the worms held on to the mucosal tissues with sufficient firmness

to ensure their survival at the height of the response. Hookworms are known to bite deep into the mucosa, penetrating the submucosa on occasion (Bonne, 1942). However, we were aware that a proportion of the worms which we recovered from concurrently infected animals were unattached (data not shown) and we do not consider that firm attachment to the mucosa is the sole explanation for our findings.

It was more surprising, but consistent with the above view, that the infective larvae of *A. ceylanicum* established without loss and grew normally when they were administered to hamsters, 8 and 14 days after infection with *T. spiralis* at the height of the response to the latter species, when mast cell counts were rising and *T. spiralis* were about to be rejected. It is again possible that these stages escaped from the direct influence of the abnormal environment in the gut mucosa and lumen by penetrating the mucosa or by entering the crypts of Lieberkuhn temporarily, as other species have been shown to do (Behnke *et al.*, 1992). However, the experimental design (involving administration of challenge inocula of hookworms either on day 8 or 14) would have ensured that in one of these combinations L4 stages, which are non-migratory, would have been in the lumen during the acute inflammatory phase. Any prolonged residence in the tissues as a means of avoiding the hostile environment on the mucosal surface and in the lumen would have been reflected in reduced growth relative to controls.

Whilst hookworms were unaffected by the presence of and the host response to *T. spiralis*, there were a number of indications that the latter species was affected by the presence of hookworms. Thus in experiments where *T. spiralis* followed an earlier infection with *A. ceylanicum*, there was a significant

reduction in the recovery of *T. spiralis*, female worms were smaller and their fecundity was depressed. Despite the statistical significance of these observations in some experiments, the magnitude of the effect on *T. spiralis* was not marked to such an extent that it would have influenced the course of intestinal inflammation. Considerably smaller worm burdens than those recorded from concurrently infected animals in this study are known to be sufficient to elicit a primary response in other rodents (Wakelin & Lloyd, 1976). On balance we conclude that the interference with the establishment of *T. spiralis* stemmed most likely from the pathological changes in the intestinal mucosa which followed infection with hookworms (Behnke, 1991b), and we do not consider these to have been a major impediment to the sequence of immunological events which ultimately culminated in the expulsion of *T. spiralis*.

A possible explanation for the chronicity of hookworm infections is that these worms, like *H. polygyrus*, are immunomodulatory and depress immunological activity in their local mucosal environment (reviewed by Behnke, 1987b). In this context, IgG antibody responses to adult *T. spiralis* antigens were depressed in concurrently infected hamsters relative to controls (Table 2) and lower serum antibody concentrations were also evident to larval antigens in animals which had harboured *A. ceylanicum* for 3–4 weeks before challenge with *T. spiralis*. However, all concurrently infected hamsters expelled *T. spiralis*, albeit marginally slower than controls in some instances, indicating that despite immunodepression the mucosal response was of sufficient intensity to ensure worm expulsion. Even in exp 6, in which marked depression of serum IgG antibody responses was observed, the expulsion of *T. spiralis* was not significantly delayed (Fig. 4). In comparison mice concurrently infected with *H. polygyrus* show a marked depression of serum antibody responses to heterologous antigens (Ali & Behnke, 1983) and delayed expulsion of *T. spiralis*, with adult worms of the latter species persisting for some 2–4 weeks after total loss from control groups infected with *T. spiralis* alone (Behnke, Wakelin & Wilson, 1978).

In conclusion, the results reported in this paper suggest that hookworms have some immonomodulatory properties and indicate that, unlike other parasitic nematodes and tapeworms, hookworms can survive the non-specific components of host-protective intestinal immune responses elicited by concurrent infections. However, the explanation for their greater reslience *vis à vis* other gastrointestinal nematodes remains to be elucidated.

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