The dynamics of trickle infections with *Ancylostoma ceylanicum* in inbred hamsters

T. J. BRAILSFORD *and* J. M. BEHNKE

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

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SUMMARY

Trickle infections with a hamster-adapted strain of the hookworm *Ancylostoma ceylanicum* were studied by administering doses of 5–30 larvae twice weekly to inbred DSN hamsters. The worm burdens were regulated at very low levels, and this was found to be independent of the size of the infective dose. It is likely that there is some turnover of adult worms during trickle infection, as larvae of all stages were recovered from most time-points. This regulation is immunologically mediated and both the worm burdens and fecundity were increased in hamsters that were immunosuppressed by cortisone treatment. A trickle infection regime also induced a 67% protective immunity to a single subsequent challenge. The resistance that occurred during trickle infections was, however, incomplete, and some worms were found to survive in hamsters that had been repeatedly infected for over 10 weeks. Thus, although hamsters are capable of regulating the infection, some worms are capable of surviving the host immune effectors.

Key words: *Ancylostoma ceylanicum*, hookworm, Nematoda, intestinal nematodes, trickle infection, repeated infection, immunity.

INTRODUCTION

Chronic infections with the human hookworms *Necator americanus* and *Ancylostoma duodenale* constitute major medical problems throughout the tropics (Banwell & Schad, 1978). Although the epidemiology of human hookworms has been extensively studied through faecal egg counts and by other techniques (Anderson & Schad, 1985; Gilles, 1985), field studies in endemic regions are problematic, not least because evaluation of the role of individual contributing factors is difficult in the face of the complex natural ecological relationships that occur among affected communities. When available, animal models provide an opportunity for controlled experimental studies through which relevant information may be derived.

The canine hookworm *Ancylostoma ceylanicum*, which also causes patent infections in man (Yoshida, Okamoto & Chiu, 1968; Areekul, Rodomyos & Viravan, 1970) has been adapted for passage through hamsters and is known to cause chronic infections reflecting many features of human hookworm disease (Ray, Bhopale & Shrivastava, 1972; Garside & Behnke, 1989). Hitherto immunological studies have focused on single pulsed primary and challenge infections which have provided evidence that acquired immunity is elicited by this parasite. They have also suggested that adult worms can evade the host effectors of resistance.

In the present paper we described repeated (trickle) infections which are more likely to provide a realistic simulation of natural infection than the usual single pulsed infections administered to experimental animals. In the past, studies of this type have provided the basis for the construction of

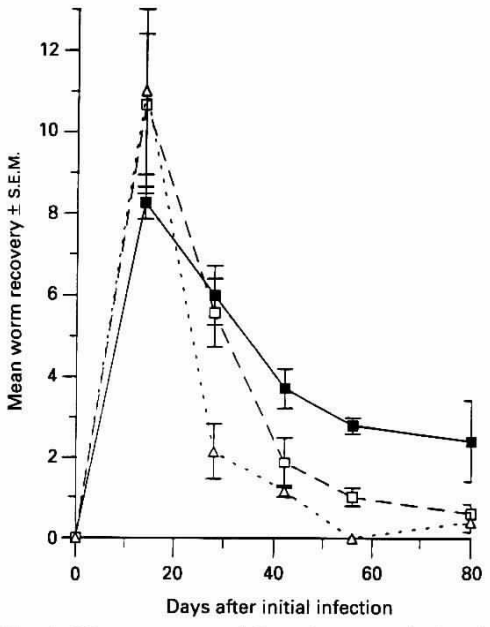


Fig. 1. Worms recovered from hamsters during the course of trickle infection with *Ancylostoma ceylanicum* (Exp. 1). The values given are the mean recovery of L3 (△), L4 (□) and adult (■) worms ± S.E.M., from hamsters infected twice weekly with 30 larvae. Infectivity controls showed a mean primary infectivity of 42·3 ± 2·99%.

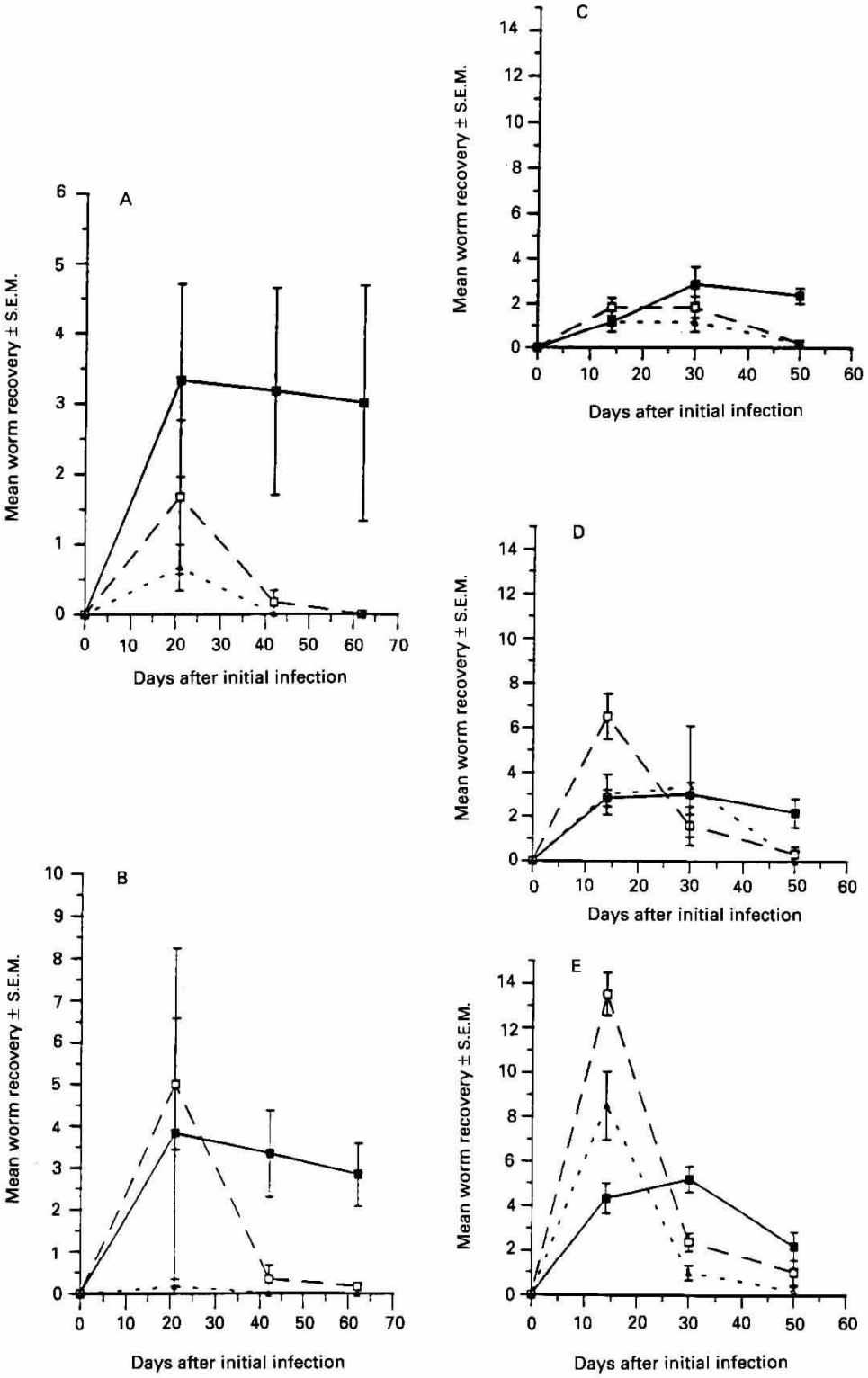


Fig. 2. Worms recovered from hamsters during the course of trickle infection with *Ancylostoma ceylanicum* (Exp. 2(A and B) and Exp. 3(C, D and E)). The values given are the mean recovery of L3 (△), L4 (□) and adult (■) worms ± S.E.M., from hamsters infected twice weekly with 5 (A and C), 15 (B and D) or 30 (E) larvae. Infectivity controls showed a mean primary infectivity of 25·0 ± 4·57 % for Exp. 2 and 49·0 ± 7·14 % for Exp. 3.

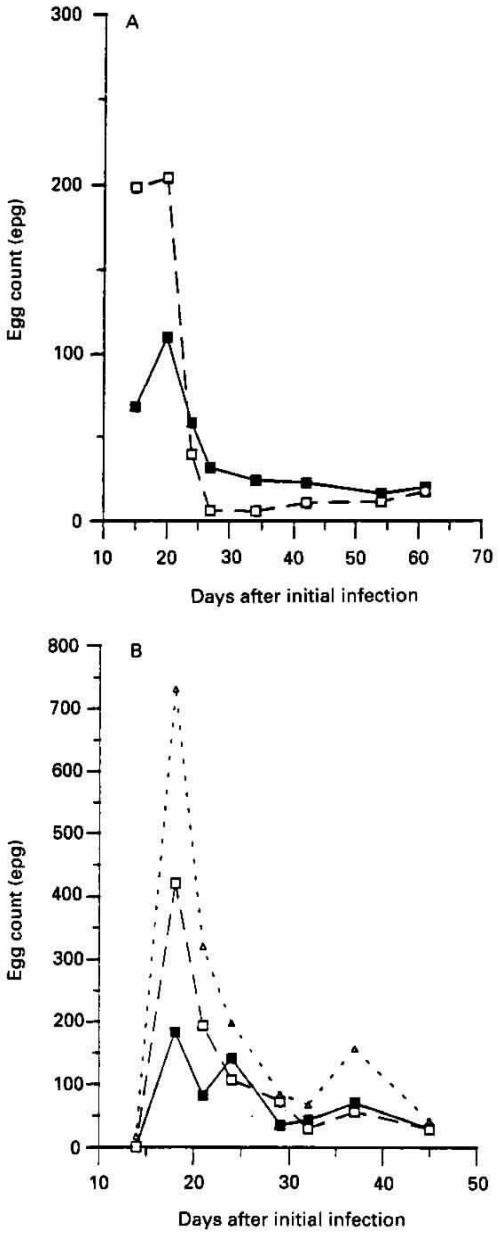


Fig. 3. Egg counts from hamsters infected twice weekly with 5 (■), 15 (□) or 30 (△) larvae, in Exps 2 (A) and 3 (B). The values given are the egg counts of faeces pooled for each group.

epidemiological models of parasite infections (Crombie & Anderson, 1985).

MATERIALS AND METHODS

Parasite

The parasite used in this study was a hamster-adapted strain of *A. ceylanicum* that has been maintained in Nottingham since 1984. The parasite was passaged every 4–6 weeks using a dose of about 50 larvae, and faeces were collected on wet paper tissue underneath wire-bottomed cages. A more efficient method of culturing than those described previously was used. The maximal yield was obtained from faeces collected 17–20 days post-infection, but reasonable numbers of larvae could be cultured from faeces collected up until 40–50 days post-infection. About 100 g of wet faeces were mixed with 5 ml amphotericin-B (fungizone), and a small amount of granular charcoal. This mixture was then added to sterilized peat in a ratio of approximately 1:3 and mixed with distilled water to make an adhesive paste. Suitable quantities of this peat–faeces mixture were then placed on moist filter paper in 9 cm plastic Petri dishes. Each dish was placed inside a larger square Petri dish and sealed with adhesive tape. These were incubated at 28 °C for 6–10 days. Infective larvae were harvested from the lids of the inner Petri dishes, washed in distilled water before use, and were either used immediately or were stored in 0·07 M phosphate-buffered 0·4 % saline for several weeks until required, with no apparent loss of infectivity.

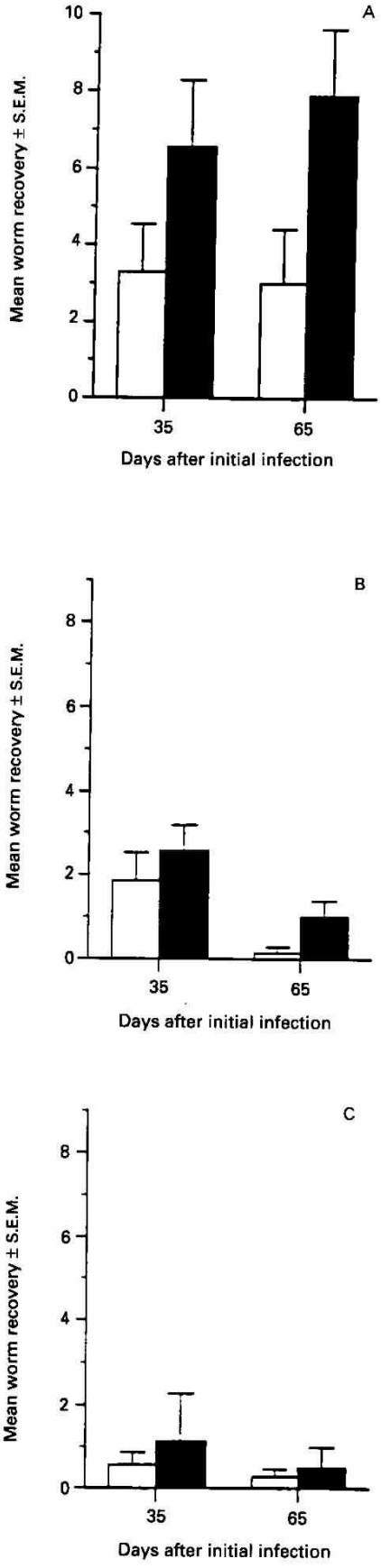
Hamsters were infected intragastrally with 0·2 ml of inoculum using a blunt needle. For trickle infections this was carried out twice weekly. To test the infectivity of larvae used for the trickle infections a few 'infectivity' controls were set up at several points throughout the experiments. These consisted of primary infections with 50 larvae, with the worms being counted at autopsy about 21 days post-infection. On autopsy the worms were recovered by opening the small intestine longitudinally and removing any worms visible, with the aid of a dissecting microscope. Gut tissues were then incubated in Hanks's saline at 30 °C for about 1 h and examined again. In order to recover pre-adult larvae, tissues were incubated in a mesh suspended in Hanks's saline at 37 °C for about 2–3 h, and the larvae were recovered by sedimentation after migrating out from the tissues. In the case of trickle-infected animals the autopsy was always carried out 4 days after the last infection. Faeces for egg counting were collected over a 24 h period and the number of eggs/g (epg) were counted by direct flotation as described by Jackson (1974).

Host

Inbred DSN hamsters (*Mesocricetus auratus*) were used throughout this study, either being purchased from Intersimian Ltd, Oxford, UK, or bred in the departmental animal house. Animals were aged 6–10 weeks at the start of experiments. Where necessary, hydrocortisone (Sigma Chemicals Ltd) was administered subcutaneously twice weekly, each animal receiving 0·2 ml containing 25 mg/ml.

Statistical analysis

Worm burdens are presented as group mean worm recovery (MWR) ± standard error (S.E.M.). Where



required data were analysed using the unified ranking system described by Meddis (1984) to test specific *a priori* hypotheses.

RESULTS

Worm burdens during the course of trickle infection

An experiment was carried out to determine the course of trickle infection in hamsters subjected to regular doses of infective *A. ceylanicum* larvae. Groups of 5–7 hamsters were infected twice weekly with doses of 30 larvae (Exp. 1), and they were killed 14, 28, 42, 56 and 80 days after the initial infection. The worm burdens obtained from these animals are given in Fig. 1. In comparison to the total number of larvae administered, only small numbers of adult worms were recovered, with a mean of $8{\cdot}25 \pm 0{\cdot}4$ after 2 weeks of the experiment, declining to $3{\cdot}7 \pm 0{\cdot}48$ by 6 weeks, and remaining fairly stable thereafter. Initially there were slightly higher numbers of L3 and L4 than adults, but these were reduced during the later stages of the experiment, although a few larvae were recovered even after 11 weeks of trickle infection. Egg counts closely reflected adult worm burdens, reaching a peak of 781 epg on day 24 and declining sharply to less than 200 by day 30. Thereafter the egg counts fluctuated between 233 and 67 epg.

The influence of number of larvae/inoculum on the course of trickle infection

Two experiments (Exps 2 and 3) were carried out to determine the influence of varying the number of larvae administered during trickle infections on worm establishment and survival. In Exp. 2, groups of 7 hamsters were infected twice weekly with doses of either 5 or 15 larvae and killed 21, 42 and 62 days after the initial infection. In Exp. 3 groups of 5–6 hamsters were infected twice weekly with doses of 5, 15 or 30 larvae, and killed 14, 30 and 50 days after the initial infection. The worm burdens for both experiments are summarized in Fig. 2, and the egg counts in Fig. 3. In Exp. 2, when the first batch of animals was killed on day 21, no significant differences were found between the worm burdens of hamster infected with doses of 5 and 15 larvae. However, dose-dependent establishment was observed in Exp. 3, 14 days after commencement of trickle infections ($L = 57{\cdot}0$, $Z = 2{\cdot}94$, $P = 0{\cdot}0019$), but there were no significant differences between the

Fig. 4. Worms recovered from hamsters infected twice weekly with 10 *Ancylostoma ceylanicum* larvae and either treated (■) or not treated (□) with cortisone (Exp 4). The values given are the mean recovery of adult (A), L4 (B) and L3 (C) ± S.E.M. Infectivity controls showed a mean primary infectivity of $26{\cdot}4 \pm 3{\cdot}78\%$.

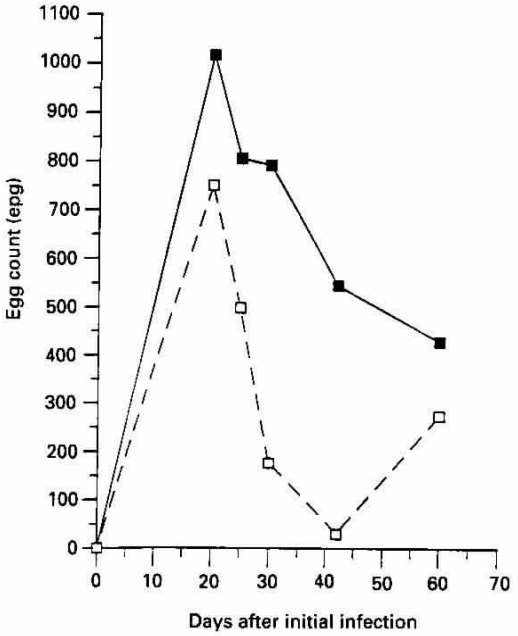


Fig. 5. Egg counts of hamsters infected twice weekly with 10 *Ancylostoma ceylanicum* (Exp. 4) and either treated (■) or not treated (□) with cortisone.

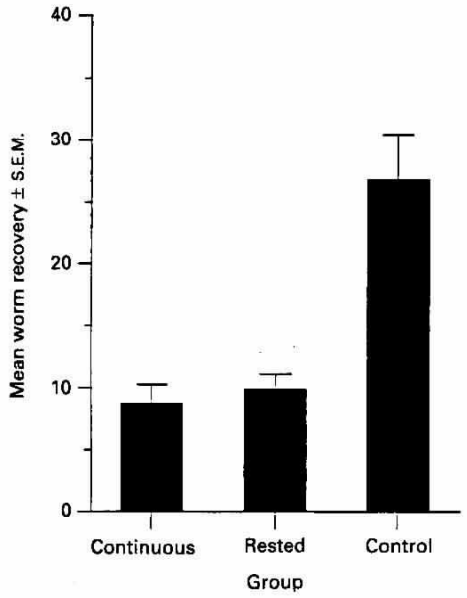


Fig. 6. Worms recovered from hamsters 21 days after a single challenge infection with *Ancylostoma ceylanicum* larvae. The 'Continuous' group had been previously infected twice weekly with 30 larvae for 50 days. The 'Rested' group had been trickle infected for 30 days and then rested for 20, and the 'Control' group were naive. Infectivity controls showed a mean primary infectivity of 33·3 ± 6·05 %.

worm burdens at subsequent autopsies on days 30 and 50. Egg counts from both of these experiments showed dose dependence during the early stages of trickle infection, but none after the first 4 weeks.

Trickle infections in immunosuppressed hosts

The potential role of immunity was investigated by comparing cortisone-treated and non-treated groups of 7 hamsters which were subjected to twice-weekly infections with doses of 10 larvae and killed on days 35 and 65 (Exp. 4). The adult worm burdens were more than doubled on days 35 and 65 in cortisone-treated hamsters compared to controls, as is shown in Fig. 4 (day 35: $L = 74$, $Z = 2{\cdot}8$, $P = 0{\cdot}0028$; day 65: $L = 91{\cdot}5$, $Z = 3{\cdot}2$, $P = 0{\cdot}0009$). There were also increased egg counts (Fig. 5), although only very slight increases in the numbers of L3 and L4 recovered were observed.

Acquired immunity elicited by trickle infection

In Exp. 5, groups of 7–8 hamsters were infected twice weekly with doses of 30 larvae. One group was infected for 30 days and then rested for 20 days. A second group was infected continuously for 50 days and a third group was not infected during this period. On day 50 all the hamsters were given a single dose of 70 larvae and were killed 21 days later for the recovery of adult worms as shown in Fig. 6 (no larvae were found in these animals). The trickle-infected group had significantly lower worm burdens following challenge infection than did age-matched controls ($L = 427$, $Z = 3{\cdot}56$, $P = 0{\cdot}003$). No difference, however, was found between the group that had been continuously infected and that which had been rested.

DISCUSSION

During trickle infections with *A. ceylanicum* in hamsters, worm burdens were regulated at very low levels throughout the course of each experiment. The size of the infection doses had little effect upon the resulting worm burdens except in the early stages of the infection, presumably before the onset of regulation (day 14, Exp. 3). The only previous experimental work involving trickle infections with hookworms is that of McCoy (1931), who repeatedly infected dogs with *A. caninum*. This work is difficult to interpret as it involved the use of very small numbers of animals in experiments of varying design. However, in some cases very low egg counts were reported from dogs regularly given large doses of larvae, suggesting that regulation of parasite numbers was taking place under trickle-infection regimes.

In more recent years trickle infections with a number of other species of gastro-intestinal nematodes have been studied, in particular the common parasites of sheep and cattle have been investigated in this context. There seem to be at least two distinct regulatory mechanisms operating to limit worm burdens during continuous infection. In the case of

Ostertagia ostertagi in cattle and *O. circumcincta* in sheep, infections are regulated by a continual turnover of adult worms and a gradual decrease in the establishment of new larvae (Michel, 1969, 1970; Waller & Thomas, 1978; Seaton *et al.* 1989*b*), although in the case of *O. circumcincta* at least, complete immunity to incoming larvae can be eventually developed. Sheep subjected to trickle infections with *Haemonchus contortus*, however, show a quite different form of regulation by means of the inhibition of larval establishment, with no significant turnover of established worms. There is an initial dose-dependent linear accumulation of parasites, followed by a period of stability. During this latter period only adult worms can be recovered. There is then a rapid dose-dependent expulsion of worms (Christie, Brambell & Charleston, 1964; Barger *et al.* 1985). A similar mechanism of regulation where incoming larvae either fail to establish or are rejected prior to the rejection of adult worms seems to occur with *Trichostrongylus colubriformis* and *T. vitrinus* in sheep and *T. axei* in cattle (Donald & Waller, 1982; Jackson, Angus & Coop, 1983; Seaton *et al.* 1989*a*).

There have also been a number of studies of the dynamics of trickle infection in laboratory models using *Nippostrongylus brasiliensis*, *Trichuris muris* and *Heligmosomoides polygyrus*. In the case of *N. brasiliensis* trickle infections only result in prolonged fecund infections in young rats where the situation is complicated by neonatal unresponsiveness (Jenkins & Philipson, 1970, 1972). In *T. muris* trickle infections a small number of worms become established following the initial infection, but none do thereafter (Wakelin, 1973; Behnke & Wakelin, 1973). *H. polygyrus* trickle infections are regulated by a means similar to that of *Haemonchus* and *Trichostrongylus* in that there is an initial linear increase of worms followed by a period of stability and then expulsion (Slater & Keymer, 1986; Maema, 1986). It is distinguished from the cattle systems, however, by the observation that during this plateau phase larvae initially establish and are expelled at a later stage in their development prior to maturation (Brailsford & Behnke, 1992).

In the present study we found a rapid onset of regulation of *A. ceylanicum* under various trickle-infection regimes in laboratory hamsters, and the resulting pattern of infection was distinct from either of the two ruminant models. There was, however, some limited evidence to suggest that there may have been a turnover of adult worms because both L3 and well-developed L4 larvae were found in small numbers at most time-points. The increased worm burdens and fecundity in the immunosuppressed hamsters indicate that regulation was immunologically mediated. The existence of acquired resistance to hookworm in humans is still controversial (Behnke, 1987), but it is well documented for *A. ceylanicum* in dogs (Carroll & Grove, 1985*a*, *b*) and in hamsters (Gupta & Kattiyar, 1985; Menon & Bhopale, 1985; Garside, Behnke & Rose, 1989). This was confirmed in Exp. 5, where, quite clearly, challenge infections were less successful in hamsters which had been previously infected than they were in naive controls.

Finally, the experiments we have reported here demonstrate that the acquisition of *A. ceylanicum* by hamsters, exposed to trickle-infection regimes attempting to mimic natural infections, was regulated by immune intervention. Quite clearly resistance was incomplete and, despite continuous exposure to larvae twice weekly, most hamsters harboured some adult worms 10 weeks after initial exposure. This finding emphasizes again that adult hookworm are capable of surviving host immune effectors, and they are likely to employ evasive strategies which have yet to be elucidated.

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