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Necator americanus* and *Ancylostoma ceylanicum*: development of protocols for dual infection in hamsters

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Summary

Two-day-old baby hamsters were infected initially with the infective larvae of hamster-adapted human hookworm, *Necator americanus* (NaL₃). After a specified period they were again infected orally with infective larvae of *Ancylostoma ceylanicum* (AcL₃). Three weeks after the second infection they were killed and the establishment of *N. americanus* and *A. ceylanicum* was assessed. The effect of different infection levels and exposure period of *N. americanus* on the concurrent establishment of *A. ceylanicum* was also examined. An infection with 50 NaL₃ percutaneously, and 3 weeks later, a second infection with 50 AcL₃ orally has produced reasonably equal number of hookworms (no statistical difference in the burden of *N. americanus* and *A. ceylanicum*) in the intestine of hamsters. Thus this protocol of dual infection was found suitable to develop two species of hookworms in hamsters for anthelmintic screening.

Key words: *Necator americanus*; *Ancylostoma ceylanicum*; monospecific infection; dual infection; hamster.

Introduction

The hookworms, *Necator americanus* and *Ancylostoma duodenale* are the commonest helminth parasites of human beings throughout the world (Woodruff, 1965; Peters, 1978; van den Bossche, 1980) and they also occur sympatrically on the Indian sub-continent and over much of the world, including parts of South America, the Far East and Africa (Hoagland and Schad, 1978). Many attempts have been made to establish them in laboratory animals (Schwartz

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and Alicata, 1934; Yoshida and Fukutome, 1967; Ray et al., 1972; Chattervati et al., 1978; Bhopale and Menon, 1979) for experimental purpose. Amongst several animals examined, the golden hamster, *Mesocricetus auratus*, seems to be the most suitable laboratory host in which the hookworm undergoes complete development. Sen and Seth (1967) have developed the human hookworm, *N. americanus*, and Ray and Bhopale (1972) a canine/human hookworm *A. ceylanicum* in hamsters. The monospecific infection of these two species of hookworms has been successfully maintained in hamsters at this laboratory over several generations to develop antihookworm drugs (Sen, 1976; Vakil et al., 1977; Doshi et al., 1977; Ray et al., 1978; Sen and Deb, 1981). So far hamsters have been infected with only one species of hookworm (either *N. americanus* or *A. ceylanicum*) and used for anthelmintic screening. Now there is a need to develop a dual infection in hamster for chemotherapeutic as well as other biological experimentations. Such a dual-infection model would allow the examination of the activity of synthetic compounds on two species of hookworm at one time. In order to meet the requirement of the pharmaceutical industry for broad-spectrum anthelmintic screening, it was felt that test animals with multiple infections would offset the cost of screening. However, it is known that when animals are infected with more than one species of parasites, competition, cross immunity and other forms of interaction may have a detrimental effect on the parasites concerned. Therefore, it was felt that a thorough investigation of the interaction of *N. americanus* and *A. ceylanicum* is required before this dual infection system could be reliably adopted in a screen for antihookworm drugs. The experiments reported here demonstrate that it is possible to develop both species of hookworms alongside each other in concurrent infections in the hamster.

Materials and Methods

Animals. The golden-hamsters, *Mesocricetus auratus*, originated from Ciba-Geigy AG, Switzerland, outbred in a closed colony without untoward effect since 1965 at Ciba-Geigy Animal Breeding Centre, Goregaon, Bombay were used. They were moved from the breeding centre to the laboratory on the day of the experiment and fed on balanced food-pellets (formulated according to National Institute of Health, USA) and water ad libitum, and were conventionally maintained throughout the experiments by changing the bedding (dry-sterilized paddy-husk) thrice weekly in autoclaved stainless/plastic (macralon) cages (measurements 41 cm × 26 cm × 15.5 cm) at $21 \pm 2^\circ\text{C}$ controlled room temperature. The animals used in the present study were the offsprings derived from the parental breeding stock used by earlier workers (Sen and Seth, 1967; Ray et al., 1978). Their age and sex are specified under each experiment.

Parasite, culture and infection. Hamsters with monospecific infection of hamster-adapted *N. americanus* or *A. ceylanicum* were used as hookworm egg donors. They were kept overnight in a cage on wire-mesh without food and the faecal pellets (the source of hookworm eggs) were collected. After soaking the pellets in water for 2 to 4 h, a faecal culture paste was prepared by emulsifying the pellets with activated granular charcoal in 3:1 proportion. Then the culture paste was transferred to and loosely filled in a 15 cm diameter corning glass petridish which was again placed in another 20 cm diameter petridish containing about 5 mm depth of distilled water. Mycostatin

solution (strength 3000 i.u./ml) was gently sprayed on the faecal culture mass. The culture was covered and maintained for 12 days at $24 \pm 2^\circ \text{C}$. The infective larvae of *N. americanus* (NaL₃) migrating to the water in the external dish were transferred to a graduated cylinder in which they were further washed in sterile distilled water 3 times by sedimentation and decantation. The washed larvae were enumerated by conventional dilution technique. Two-day-old baby hamsters were infected percutaneously under anaesthesia with the required number of NaL₃ in 20 μl .

The faecal cultures of *A. ceylanicum* were set up. After 12 to 15 days of culturing the infective stages of *A. ceylanicum* (AcL₃) were harvested by Baermann apparatus in water and then washed and counted. The required number of infective larvae was taken in 0.5 ml water and each hamster was infected per os with this inoculum.

The age and sex of hamsters; dose, type and period of infection and the time of killing are specified under "Results".

Assessment of development and distribution of N. americanus and A. ceylanicum. Hamsters infected once or twice with *N. americanus* and *A. ceylanicum* were killed in excess ether. The small intestine was removed and opened lengthwise in 9 cm diameter petridish with 25–30 ml of 0.85% NaCl solution (normal saline). Initially the undigested material and the debris were cleared away from the intestine and the parasites which were free in the lumen collected. Then, the intestine was scraped and all parasites present in the scrapings were picked with fine forceps by examining under Carl-Zeiss stereo dissection microscope. Parasites collected from each intestine were washed 3 times in a 5 ml beaker with warm (37°C) buffered balanced salt saline (BSS) pH 7.4 and identified morphologically under a Leitz microscope. In this way the number of *N. americanus* and *A. ceylanicum* developed in the hamsters was determined.

In one experiment the distribution of *N. americanus* and *A. ceylanicum* in the small intestine was examined. The entire small intestine and stomach was removed and spread lengthwise on a glass piece with metric scale on the background. The intestine was split open lengthwise using a fine pair of scissors and was spread along the metric scale. The worms were individually picked, identified under microscope and their location was noted down. In this way all worms present in the small intestine were collected and the exact location of *N. americanus* and *A. ceylanicum* in the small intestine was determined.

Results

Development of A. ceylanicum in hamsters infected previously with different levels of N. americanus

Only 1- to 2-day-old baby hamsters can be infected with *N. americanus* whereas the weanlings and a few-week-old ones are refractory to infection (Sen and Seth, 1967). Moreover about 40–50% of infected baby hamsters die before weaning due to various reasons. Comparatively hamsters up to 4 months old are known to be susceptible to *A. ceylanicum* (Ray and Bhopale, 1972). With these limitations, attempts were made to establish a dual infection of *N. americanus* and *A. ceylanicum* (Na + Ac) in hamsters. A uniform protocol was followed in experiments described below: 1. Two-day-old hamsters were infected initially with different levels of NaL₃ percutaneously. 2. Three weeks later surviving infected baby hamsters were weaned, grouped and further infected with different levels of AcL₃ orally. 3. Equal number of hamsters were infected either with NaL₃ or with AcL₃ at the appropriate time as single infection controls. 4. Ten animals were kept in each group. 5. Three weeks after second infection with AcL₃ all animals were killed and worms were collected and identified.

Different combination of dosages were administered to choose an optimum infection dosage of NaL₃ and AcL₃ which produce a satisfactory burden of *N. americanus* and *A. ceylanicum*. Many experiments were performed. Results obtained can be summarized as follows: 1. A dual infection of 75 NaL₃ + 75 AcL₃ has resulted about 30 to 50% mortality. The surviving hamsters had mean (\pm SD) burden of 4.3 ± 4.6 *N. americanus* and 38.8 ± 9.4 *A. ceylanicum*. 2. In 25 NaL₃ + 25 AcL₃ infection schedule all hamsters survived with a mean (\pm SD) of 4.7 ± 4.2 *N. americanus* and 2.6 ± 2.7 *A. ceylanicum*. This worm burden was regarded as very low for chemotherapeutic studies and moreover some of the hamsters were free from either *N. americanus* or *A. ceylanicum*. 3. A dual infection of 20 NaL₃ + 50 AcL₃ has resulted a mean (\pm SD) of 6.5 ± 5.6 *N. americanus*. Only two hamsters carried above 10 *N. americanus* and the remainder either had less than 10 or no worms at all. In contrast a mean (\pm SD) of 5.5 ± 2.8 *A. ceylanicum* were developed and none of the hamsters were free from infection at this dosage. Then 4. a dosage of 50 NaL₃ + 50 AcL₃ was administered.

Results of two experiments performed are shown in Table 1 and summarized as follows: 1. No animal was free from any one type of hookworm parasite. 2. Except two, other individual animals carried more than 10 worms in dual infection group. 3. Variation was apparent in the establishment of a particular type of hookworm in individual hamsters in dual infection groups although worm establishment was not significantly different ($p > 0.05$) from the respective single-infection controls. 4. Variation between *N. americanus* and *A. ceylanicum* was marked in some animals with the burden of a particular species 2 times or 3 times greater than the other. However, 5. competition between two species was not evident when their development in dual infection group was compared with their respective single infection controls.

Distribution of adult N. americanus and A. ceylanicum in small intestine of hamsters

It is clear that both *N. americanus* and *A. ceylanicum* (Na + Ac) can be established together in individual hamsters. As the habitat of two species remains the same it is interesting to investigate whether the existing *N. americanus* infection influence the establishment of on-coming *A. ceylanicum* larvae. To examine this, three groups of hamsters were employed; two were infected with single species (either *N. americanus* or *A. ceylanicum*) and the third one with dual infection of Na + Ac. Infection doses, interval between infections and killing of animals were similar to previous experiments. Fig. 1 shows the distribution of *N. americanus* and *A. ceylanicum* in single as well as doubly infected animals. The majority of worms, irrespective of species, were recovered in the region ranging from 3 to 22 cm distance from the stomach. The site occupied by the hookworms was found to be the same in both single as well as in doubly infected animals and also in those with comparatively heavier worm-load. A

Table I. Dual infection of *N. americanus* and *A. ceylanicum* in hamster

| Group | Parasite infection* | | No. of hamsters survived/used | Worm recovery** <i>N. americanus</i> (Na) and <i>A. ceylanicum</i> (Ac) | | Mean \pm SD Δ |
|-------|----------------------------|----------------------------|----------------------------------|--|------------------|------------------------|
| | <i>N. ameri- canus</i> | <i>A. ceyla- nicum</i> | | Worms from individual hamsters | | |
| I A | + | — | 9/9 | Na 10, 11, 15, 15, 17, 20, 20, 21, 36 | 18.3 \pm 7.7a | |
| I B | + | + | 8/10 | Na 12 13 20 27 27 29 29 30 | 23.4 \pm 7.4b | |
| | | | | Ac 39 19 15 13 21 10 12 31 | 20.0 \pm 10.2c | |
| I C | — | + | 8/10 | Ac 6, 8, 12, 20, 21, 28, 29, 30 | 19.3 \pm 9.6d | |
| II A | + | — | 10/10 | Na 5, 6, 10, 11, 12, 12, 13, 15, 16, 20 | 12.0 \pm 4.5e | |
| II B | + | + | 8/10 | Na 7 9 10 11 13 17 22 22 | 13.9 \pm 5.8f | |
| | | | | Ac 14 30 31 29 12 38 16 21 | 23.9 \pm 9.4g | |
| II C | — | + | 8/11 | Ac 9, 14, 14, 16, 17, 20, 24, 34 | 18.5 \pm 7.7h | |

* 50 *N. americanus* infective larvae (NaL₃) were administered percutaneously when hamsters were 2 days old and 50 *A. ceylanicum* (AcL₃) per os three weeks later.

** Hamsters were killed 3 weeks after infection with AcL₃ and worms collected from the small intestine.

$^{\Delta}$ Statistical analysis: b vs a, c vs d, e vs f, g vs h: p > 0.05 NS (Mann-Whitney U-test).

Table 2. Effect of *N. americanus* infection on the establishment of *A. ceylanicum* in hamster

| Group | Parasite infection | | No. of hamsters survived/used | Worm recovery ^Δ <i>N. americanus</i> (Na) and <i>A. ceylanicum</i> (Ac) | | | | | Mean ± SD ^{ΔΔ} |
|-------|------------------------------|-------------------------------|----------------------------------|---|----------------------------------|--|--|--|-------------------------|
| | <i>N. ameri- canus</i> * | <i>A. ceyla- nicum</i> ** | | Worms from individual hamsters | | | | | |
| | | | | | | | | | |
| I A | + | — | 4/4 | Na | 0, 3, 11, 23 | | | | 9.3 ± 10.3a |
| I B | + | + (week 6) | 5/5 | Na | 6 7 12 12 13 | | | | 10.0 ± 3.2b |
| | | | | Ac | 0 3 5 25 30 | | | | 12.6 ± 13.8c |
| I C | — | + | 9/9 | Ac | 4, 7, 14, 16, 17, 25, 26, 27, 27 | | | | 18.1 ± 8.8d |
| II A | + | — | 6/6 | Na | 0, 5, 5, 7, 9, 14 | | | | 6.7 ± 4.7e |
| II B | + | + (week 9) | 8/9 | Na | 4 5 6 10 12 12 12 16 | | | | 9.6 ± 4.2f |
| | | | | Ac | 1 0 7 1 0 1 12 0 | | | | 2.8 ± 4.4g |
| II C | — | + | 7/10 | Ac | 11, 14, 17, 20, 34, 39, 41 | | | | 25.1 ± 12.5h |

* Infection given to 2-day-old hamsters each with 50 NaL₃ percutaneously.
 ** Six or 9 weeks after infection with *N. americanus*, each hamster received 50 AcL₃ orally.
 Δ Hamsters were killed 3 weeks after infection with AcL₃.
 ΔΔ Statistical analysis: a vs b, c vs d, e vs f, a vs e, c vs g, d vs h: p > 0.05 NS; g vs h: p < 0.01 (Mann-Whitney U-test).

few *A. ceylanicum* were recovered from the distal regions of the intestine but none beyond 35 cm.

Effect of duration of N. americanus infection on the establishment of A. ceylanicum in hamster

In earlier experiments an interval of 3-week duration was uniformly maintained between the infections with *N. americanus* and *A. ceylanicum*. It was of interest to see whether there would be any variation in the degree of establishment of *A. ceylanicum* in hamsters with an extended period of *N. americanus* infection. To examine this, infected hamsters were given a second infection of *A. ceylanicum* 6 and 9 weeks after *N. americanus* infection. Animals were killed 3 weeks after *A. ceylanicum* infection and all the worms were collected from small intestine and identified. The results are shown in Table 2. It can be pointed out that 1. in singly infected groups two hamsters were free from *N. americanus* (IA and IIA) whereas 2. all animals had *A. ceylanicum* (IC and IIC); 3. in doubly infected groups (IB and IIB) the “take” of *A. ceylanicum* was highly variable (range 0–30) during 6- and 9-week period.

Discussion

Attempts were made to establish dual infections of *N. americanus* and *A. ceylanicum* in the small intestine of hamsters for chemotherapeutic as well as other biological purposes. This approach was adopted to overcome the limitations imposed by the fact that *N. americanus* develops only in newborn baby hamsters (Sen and Seth, 1967) whereas in contrast, hamsters as old as 4 months are susceptible to *A. ceylanicum* (Ray and Bhopale, 1972). In view of this, experiments were initiated with 2-day-old baby hamsters receiving *N. americanus* infection percutaneously and 3, 6 and 9 weeks later *A. ceylanicum* orally. Several infection doses of *N. americanus* and *A. ceylanicum* were used. Of which the infection of baby hamsters initially with 50 NaL₃ percutaneously, 3 weeks later, 50 AcL₃ orally was found to be the ideal protocol to establish a dual infection of Na + Ac. Under these conditions a range of 28–47% of NaL₃ and 40–48% of AcL₃ were developed into adult worms in doubly infected animals. This “take” in doubly infected animals was nonetheless comparable with single infection controls (a range of 24–37% NaL₃ and 37–39% AcL₃) and found consistent when second infection with AcL₃ given 3 weeks later. When AcL₃ were given 6 and 9 weeks after NaL₃ infection, the development of these two species was somewhat disturbed. The burden of *N. americanus* was lesser both in single as well as in doubly infected animals during 6- and 9-week pre-exposure period (Table 2) compared to previous experiments (Table 1). The establishment of *A. ceylanicum* was significantly disturbed in doubly infected hamsters. Some hamsters were free from *A. ceylanicum* and about 30% and 89% reduction in the development of *A. ceylanicum* was observed during 6- and 9-week pre-exposure

period respectively (Table 2). This indicates that a degree of cross resistance appears against the establishment of *A. ceylanicum* in previously infected animals after 6-week pre-exposure with *N. americanus*. This cross resistance does not appear to build up, if second infection given about 3 weeks after the first. Thus the interval between two infections seems critical in establishing two hookworm species. However, under the present experimental conditions an interval of 3-week period between infections seems ideal to obtain a satisfactory adult worm burden of two hookworms in hamsters as *N. americanus* reach maturity between 35 and 40 days (Sen and Seth, 1967) and *A. ceylanicum* 16 days after infection (Ray and Bhopale, 1972).

Thus this dual model, despite a few limitations, would be of some use for examining the activity of synthetic compounds on two hookworm species at one time and also for studying the pathogenesis and clinical picture of sympatrically occurring *N. americanus* and *A. duodenale* infection in humans (Hoagland and Schad, 1978; WHO, 1981).

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