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Site of resistance to *Necator americanus* in hamsters*

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Summary

Resistance to the development of human hookworm, *Necator americanus* was examined in 3- to 6-week-old young adult hamsters. Only 3% of *N. americanus* infective third stage larvae (NaL_3) reached maturity in the intestines of young adults as opposed to as many as 60% in 2-day-old baby hamsters. This seemingly effective resistance prevailing in young adults was investigated in some detail. The skin, the first site of contact for the invading NaL_3, was by-passed during the infection process. Completely in vitro exsheathed NaL_3 ($ExNaL_3$) were used, and young adult hamsters were infected parenterally, by-passing the skin. Even after exsheathing the larvae artificially before infection and by-passing the skin, no improvement was seen in the development of *N. americanus* in the intestines of young adults. Higher infection doses also did not increase the worm burden. Some of the factors limiting the development of parasites in young adults were examined. *N. americanus* were monitored in lungs and intestines during various intervals after infection. Similar parasite burdens were apparent in lungs of baby as well as young adult hamsters. In the intestines, a significantly lower burden of *N. americanus* was seen during various intervals in young adults compared to the baby hamsters. Moreover, *N. americanus* were expelled soon after reaching the intestine. This comparative monitoring revealed the intestine as the seat of resistance against the establishment of *N. americanus* in young adult hamsters.

Key words: *Necator americanus:* in vitro exsheathment; baby hamsters; young adult hamsters; age resistance; immunology; tissue migration; worm recovery; lungs; intestine

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Introduction

There is no suitable animal model with which to study the immunology of the human hookworm, *Necator americanus*. Sen and Seth (1967) used 2-day-old baby hamsters which are susceptible and in which *N. americanus* undergoes complete development. However, not many immunological studies are possible in baby hamsters because they have to be infected as early as 2 days after their birth. Moreover, as many as 40% of infected babies die due to several reasons (Rajasekariah et al., 1985). Three- to 6-week-old young adult hamsters are therefore preferred but they are generally regarded as "resistant" (Ogilvie et al., 1975). However, no comparative data are available on the extent of establishment of *N. americanus* in hamsters of different age groups. In view of the limitations in using baby hamsters, there is a need to study the natural resistance and to pinpoint the possible site(s) of resistance against the establishment of *N. americanus* in young adult hamsters. Some experiments conducted on these lines are reported here.

Materials and Methods

Hamsters. Two-day- (baby) and 3- and 6-week-old (young adult) golden hamsters (*Mesocricetus auratus*) were used for infection. After infection, baby hamsters were maintained with their mothers until weaning on day 21. The maintenance and handling of hamsters during the period of study are described elsewhere (Rajasekariah et al., 1985).

Harvesting ensheathed infective larvae of N. americanus ($EnNaL_3$): their exsheathment in vitro and infection of hamsters. The production of ensheathed infective third stage larvae of *N. americanus* ($EnNaL_3$) is described elsewhere (Rajasekariah et al., 1985). The exsheathment of $EnNaL_3$ was performed in vitro. $EnNaL_3$ were washed 3 times in tissue culture medium (RPMI 1640, GIBCO, with Hepes and bicarbonate buffer pH 7.2, antibiotics and supplemented with 1% glucose) and then transferred into a glass tube (diameter 1.5 cm, length 10 cm) one end of which was closed by tying the freshly dissected skin of 3-week hamsters on to its outer rim. Care was taken to avoid any leakage. The tube containing known numbers of $EnNaL_3$ in 2 ml medium was suspended with a clamp into an external container (25 ml glass beaker) with 10 ml of medium with the closed end of the tube covered with skin fully immersed in the medium. The whole system was incubated at 37° C for 24 h under sterile conditions. During the incubation period $EnNaL_3$ penetrated the skin, completely exsheathed and were liberated into the external container. The exsheathment was checked under the microscope and the larvae in the external container designated "exsheathed NaL_3" ($ExNaL_3$). $EnNaL_3$ and $ExNaL_3$ were washed briefly 3 times in warm (37° C) tissue culture medium. Active larvae were counted on a heat-stage Leitz microscope and used for infection. Baby hamsters were infected percutaneously under anaesthesia (Sen and Seth, 1967). Young adult hamsters were infected 1. percutaneously by exposure to $EnNaL_3$ suspended in distilled water in a wide-mouth screw-cap bottle and 2. by administering $EnNaL_3$ intraperitoneally, intrathoracically and per os.

Assessment of development of N. americanus in hamsters. The number, type and the dose of NaL_3 used for infection and the time of sacrificing infected hamsters are mentioned in the Results section. NaL_3 were recovered from the lungs 4 to 6 days after infection. The lungs from each animal were chopped into 2 mm cubes and transferred on to a sieve (mesh size 1 mm^2) which fitted into a glass dish containing 25 ml sterile tissue culture medium. They were incubated at 37° C for 3–4 h (during incubation, the larvae migrate from the tissue and settle to the bottom of the dish). After

Table 1. Development of *Necator americanus* in naive baby and young adult hamsters

Hamster		Generation number of NaL_3*	No. of *N. americanus* collected from the small intestine of hamsters 37 days after infection each with 100 $EnNaL_3$ percutaneously		Development
Age	No. survived/ No. used		Individual recovery	Mean ± SD	
Baby	15/25	HS-68	18, 24, 28, 34, 35, 38, 41, 45, 47, 50, 60, 65, 66, 69, 79	46.6 ± 18.0[a]	62%
Young adult	10/10	HS-68	0, 0, 1, 1, 1, 1, 2, 3, 5, 9	2.3 ± 2.8[b]	3%

* NaL_3 used were derived from the 68th generation of human hookworm, *N. americanus*, adapted to hamster
Statistical analysis: b < a; P < 0.001 (Mann-Whitney U-test)

Table 2. Numbers of $EnNaL_3$ migrated through the skin of young adult hamsters during different intervals in vitro

Skin No.	No. of $EnNaL_3$ transferred on to the upper surface of the hamster skin	No. of $ExNaL_3$ collected at different intervals (in hours) during incubation in vitro					Total No. of $ExNaL_3$ recovered from each skin
		1	4	6	8	20	
Experiment 1							
Skin 1	2000	0	250	50	250	50	600
Skin 2	2000	0	100	50	50	50	250
Skin 3	2000	0	200	50	50	300	600
Experiment 2							
Skin 4	2000	0	100	65	380	130	675
Skin 5	2000	0	16	50	365	265	695
Skin 6	2000	0	65	65	415	330	875
	Mean ± SD	0	122 ± 87	55 ± 8	252 ± 166	188 ± 127	615 ± 206
	Exsheathment		6.1%	2.8%	12.6%	9.4%	30.4%

removing the sieve along with the lung pieces, the medium was examined under a Carl-Zeiss stereo dissection microscope and larvae were collected with a finely-drawn pasteur pipette. The number of exsheathed NaL_3 recovered from the lungs of each animal were counted. NaL_4 and pre-adults were recovered from the small intestines on day 12 and 27 post-infection, respectively. The intestines were opened lengthwise and chopped into pieces of about 4–5 cm in petridishes (9 cm diameter) containing about 20 ml tissue culture medium. They were subsequently incubated at 37° C for 3–4 h. Many *N. americanus* larvae dislodged themselves from the intestine during incubation and were collected. Further the intestine pieces were scraped and all larvae collected. The number of larvae (NaL_4 and pre-adults) recovered from the intestine of each animal was counted. In some experiments, pre-adult *N. americanus* were collected from intestines 37 days after infection. The adult *N. americanus* were collected from the intestines 40 days after infection.

Statistical analysis. The development of *N. americanus* in young adult hamsters was compared with that of baby hamsters. The tabulated data were analysed wherever necessary using the Mann-Whitney U-test.

Results

Comparative development of N. americanus in baby and young adult hamsters

Results in Table 1 show that about 62% $EnNaL_3$ develop in baby hamsters compared to as few as 3% in young adults. About 40% of infected babies died and all surviving ones carried significantly higher worm burdens. Compared to baby hamsters, 80% of the young adults carried significantly lower worm burdens.

Infection of young adult hamsters with $ExNaL_3$

The inability of $EnNaL_3$ to penetrate the skin could be one reason for a lower worm burden in young adult hamsters. We felt that the infection rate could be increased if the skin is by-passed. $EnNaL_3$ exsheath while penetrating the skin. Our intention was to obtain such larvae freed from the sheath so that they could be injected parenterally. A method was developed, and $EnNaL_3$ were exsheathed. An average of about 31% $EnNaL_3$ were completely exsheathed and liberated into the external container (Table 2). They were designated exsheathed infective third stage larvae ($ExNaL_3$) and used for infection.

The numbers of *N. americanus* recovered from the intestines of young adult hamsters following infection with different doses as well as different routes are shown in Table 3. *N. americanus* did not establish well even after exsheathing and injecting parenterally. The worm burden remained less than 10 per hamster, and the percentage of infectivity varied significantly. Higher infection doses also did not improve the establishment of the parasite. Three- and 6-week-old hamsters carried similar worm burdens (Table 3).

Monitoring the migration of N. americanus in lungs and intestines of baby and young adult hamsters

Migration of different developmental stages of *N. americanus* was monitored in baby and young adult hamsters to pinpoint the possible factor(s) con-

Table 3. Development of *Necator americanus* in naive young adult hamsters exposed to $ExNaL_3$

Age of hamsters on the day of infection	Infection dose No. of $ExNaL_3$ per animal	Route of infection*	No. of hamsters found infected/ No. exposed	Infection %	No. of *N. americanus* collected from small intestine of hamsters 40 days after infection	
					Individual recovery	Mean ± SD
Experiment 1						
3 weeks	50a	SC	1/5	20	2	0.4 ± 0.8
	50b**	SC	4/6	67	1, 1, 2, 2	1.0 ± 0.9
	50c	IP	5/10	50	1, 1, 1, 2, 2	0.8 ± 0.8
	50d	IT	0/6	0	none	0
6 weeks	50a	SC	5/5	100	1, 2, 3, 4, 8	3.6 ± 2.7
	50b**	SC	2/5	40	1, 2	0.6 ± 0.6
	50c	IP	0/5	0	none	0
	50d	IT	1/5	20	1	0.2 ± 0.4
Experiment 2						
3 weeks	100	SC	4/9	44	1, 2, 3, 5	1.4 ± 1.8
	500	SC	4/14	28	1, 4, 4, 5	1.0 ± 1.8
	2000	SC	3/3	100	1, 4, 6	3.7 ± 2.5

* SC = subcutaneous; IP = intraperitoneal; IT = intrathoracic

** Equal numbers of hamsters were dosed orally with $ExNaL_3$ but none developed

a, b, c, d: Experiments were conducted at the same time with identical batches of $ExNaL_3$

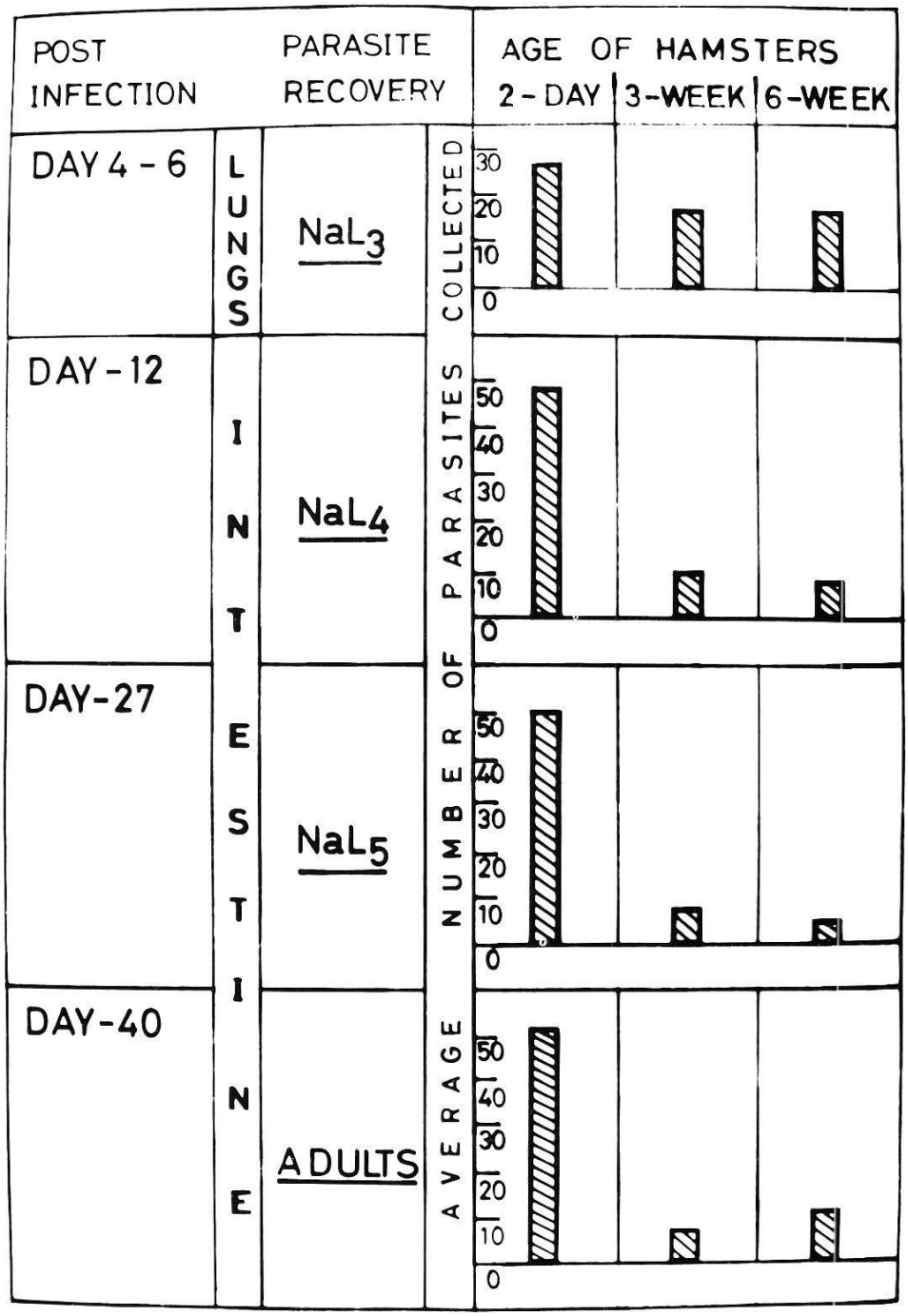

Fig. 1. Recovery of different developmental stages of *N. americanus* from baby and young adult hamsters at different intervals after infection with 100 $EnNaL_3$ percutaneously. Bars represent the average number of parasites collected at each point. The worm burdens of young adult (3- and 6-week-old) groups were not statistically different from baby hamsters in lungs ($P > 0.05$) but in the intestines young adults carried significantly lower worm burden ($P < 0.05$) than that of baby hamsters during all intervals.

trolling their development in adult hamsters. In a preliminary experiment, we recovered in vivo exsheathed NaL_3 from the lungs 4 to 6 days after infection. NaL_4 and pre-adult stages were collected from intestines 12 and 27 days after infection, respectively. Adult (male and female) *N. americanus* were collected

from the intestine 40 days after infection. Two sets of experiments were conducted. In each set 10 hamsters of each age group (2-day-, 3- and 6-week-old) were used and each received 100 $EnNaL_3$ percutaneously. Two animals from each age-group were sacrificed on day 4, 6, 12, 27 and 40 PI. Their lungs and intestines were incubated and *N. americanus* were recovered. No parasites were recovered from lungs 12 days after infection. Intestines were free from parasites up to 6 days PI. This observation was uniform in all hamsters irrespective of their age group. The data from 4 animals at each time point were pooled and shown in Fig. 1. For convenience, parasites recovered from lungs on 4 and 6 days PI ($n = 8$) were pooled and the data are shown in Fig. 1. In lungs, about 17% of larvae were recovered from young adults as opposed to about 27% in babies. This was not statistically significant ($P > 0.05$). However, young adults ($n = 4$) carried significantly lower worm burdens in the intestines 12, 27 and 40 days after infection ($P < 0.05$ at all time points) than baby hamsters. Thus, a significant difference between young adults and baby hamsters was found only for worm burdens in the intestines.

Discussion

As judged from the worm burden in the intestines, only 3% of NaL_3 developed in young adult hamsters whereas in babies, the development was as high as 60%. From these results, it is apparent that young adults are resistant to the establishment of *N. americanus*. In order to assess the possibility of using young adults as experimental hosts for immunological studies the nature of age resistance needs to be understood. The main objective was to induce higher worm burdens in adults. It was thought that the skin may act as a barrier and therefore it was by-passed. Moreover, in vitro exsheathed larvae were used for infection. Whatever the parenteral route adopted for infection, there was no improvement in the establishment of *N. americanus*. Even higher infection doses did not improve the worm burden. It could be argued that in vitro exsheathment might have affected the larval infectivity. Baby hamsters were infected with $ExNaL_3$ prepared from young adult hamster skin. Six days after infection, lungs of infected babies were examined and an average of 24% larvae were recovered (unpublished observation). We have evidence to show that $ExNaL_3$ are infective even after passing through the hamster skin.

At this stage, it is reasonable to inquire into the fate of injected larvae, and the inability of greater numbers of $ExNaL_3$ to reach maturity in young adults. It is possible that larvae might have been killed en route to the intestine. To prove this either histopathology or larval monitoring could be performed. Applying the latter we found that in the lungs, babies as well as young adults had similar worm burdens. In the intestines, however significant differences were seen in the worm burdens; young adults carried about 10 worms each compared to about 50 worms in baby hamsters.

Examination of the migration of *N. americanus* from the lungs into the intestine revealed that young adult hamsters expelled the majority of the parasites from the intestine accounting for the lowered worm burden observed. Furthermore, the lungs of babies as well as young adults were free from parasites 12 days after infection which substantiates the worm expulsion from intestines of young adults.

Comparative monitoring of worms indicated the intestine as the site of resistance. However, the nature and the mechanisms of resistance against the establishment of *N. americanus* in young adults are not yet clearly demonstrated. Kiyono et al. (1983) showed the immunological involvement of the gut-associated lymphoreticular tissues. The role played by mucosal mast cells and biogenic amines in the expulsion of gut nematodes is known. Moreover, in aged hamsters, a high level of IgA (Smith et al., 1983) and potent killer cell activities (Dutta et al., 1979; Haddada et al., 1980) have been reported. It will be relevant to pinpoint which of the resistant factors are directed against *N. americanus* and whether they are similar to the ones mediating the spontaneous expulsion of *Nippostrongylus brasiliensis* (Miller and Nawa, 1979; Levy and Frondoza, 1983) and other helminths (Castro, 1981).

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