A second peak of egg excretion in *Strongyloides ratti*-infected rats: its origin and biological meaning

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SUMMARY

In *Strongyloides ratti*-infected rats, 2 peaks of egg excretion were observed; a large one with maximum egg production on days 7–8 of infection and a small more inconspicuous one around day 25. The second peak, which had been ignored in most studies, was produced by adults in the caecum and the colon. The adults were larger in length and had more embryonated eggs in the uterus compared with adults in the small intestine at day 25 post-infection. It is suggested that parasitic adults once expelled from the small intestine resettle and recover in the large intestine. Filter paper faecal culture carried out for 9 days at different days post-infection revealed that the total number of infective larvae that developed during the second peak was twice the number that developed during the first peak, despite the fact that total egg output during the second peak was less than one twentieth of the first peak. The results suggest that the small second peak was as important as the first one in the transmission of *S. ratti*.

Key words: *Strongyloides ratti*, egg excretion, transmission, caecum, colon.

INTRODUCTION

Strongyloides ratti has been used as a model parasite of human strongyloidiasis. Being different from the parasite of humans, *S. ratti* is expelled from host animals approximately 2–3 weeks post-infection, and the mechanisms of expulsion have been studied extensively (Nawa *et al*. 1988; Uchikawa, Ichiki & Komaki, 1991; Abe *et al*. 1992; Abe, Sugaya & Yoshimura, 1998). In these studies, expulsion signified the dislodging of parasitic adults from the small intestine. However, few studies have investigated any involvement of the large intestine in the process of worm expulsion. As far as we are aware, only Abe, Yoshimura & Nawa (1993) reported the transient retention of adults in the caecum of mice. Meanwhile, the number of eggs excreted from infected animals showed a rapid rise then fall coinciding with the establishment of the infection and expulsion of adult worms. In addition, a second inconspicuous peak was sometimes observed. For example, Moqbel & Denham (1977) recorded a subtle increase in the number of excreted eggs at day 22 of infection. The second peak was so small that, in most studies, it was completely ignored. As the appearance of the second peak is a consistent finding, we decided to investigate its origin and biological significance. This has lead to the conclusion that the second peak is actually as important as the first one in the transmission of *S. ratti*.

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MATERIALS AND METHODS

Parasites and animals

S. ratti used in this study was a strain isolated from a wild *Rattus norvegicus* caught in Nagoya, Japan, in 1985 and subsequently maintained in Sprague-Dawley rats at the Department of Medical Zoology, Nagoya University. The strain was donated to this laboratory in 1990 and has been maintained in inbred male Wistar rats. For maintenance, infective 3rd-stage larvae (L3s) obtained on the 3rd to 5th day of faecal culture at 25 °C were inoculated subcutaneously every 25–35 days.

In this experiment, all animals used were commercially available inbred male Wistar rats 6 weeks of age (Japan SLC). They were kept individually in a cage with a wire-mesh bottom which allowed animal faeces to pass through, preventing reinfection of the animals. The L3s used for infection were obtained by 4-day culture of stool, which was collected from rats infected 5 or 6 days previously.

Counting the number of eggs and recovering adult worms

A group of 5 rats was infected with 3000 saline-washed L3s (Group I) and another group of 6 rats was infected with 8000 L3s (Group II). The total number of eggs and subsequently hatched rhabditiform larvae per 12 mg of stool was counted at various days post-infection for up to 88 days using a direct faecal smear method. In order to study the distribution of adult worms in the intestine, 20 rats

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Table 1. Recovery of adult worms from different parts of the intestine or days of infection

(Average of 4 animals; 3000 L3s inoculated per animal.)

Site of recovery		Day after infection				
		9	14	19	25	35
S-1	No. of worms (S.D.)	1024·3 (173·4)	837·3 (138·2)	352·8 (135·8)	69·8 (50·3)	1·8 (3·5)
	%*	43·88	44·38	25·50	8·18	0·53
S-2	No. of worms (S.D.)	970·0 (149·0)	720·3 (124·1)	183·0 (51·1)	24·3 (25·7)	0·5 (1·0)
	%	41·55	38·18	13·23	2·85	0·15
S-3	No. of worms (S.D.)	327·8 (129·4)	320·3 (91·3)	80·5 (35·1)	14·5 (18·0)	0·0 (0·0)
	%	14·04	16·98	5·82	1·70	0·00
S-4	No. of worms (S.D.)	11·3 (3·3)	8·3 (4·2)	229·0 (174·1)	39·5 (34·8)	7·8 (8·0)
	%	0·48	0·44	16·55	4·63	2·30
Cc	No. of worms (S.D.)	1·0 (1·4)	0·5 (0·6)	434·5 (285·9)	265·5 (82·0)	94·5 (13·2)
	%	0·04	0·03	31·41	31·11	27·85
C-1	No. of worms (S.D.)	0·0 (0·0)	0·0 (0·0)	103·0 (83·7)	394·3 (98·2)	214·0 (97·2)
	%	0·00	0·00	7·45	46·20	63·07
C-2	No. of worms (S.D.)	0·0 (0·0)	0·0 (0·0)	0·5 (0·6)	45·8 (19·3)	20·8 (17·5)
	%	0·00	0·00	0·04	5·37	6·13
Total	No. of worms (S.D.)	2334·3 (380·0)	1886·5 (206·7)	1383·3 (534·5)	853·5 (202·6)	339·3 (121·4)
	%	100·0	100·00	100·00	100·00	100·00

* Percentage of the total.

Table 2. Body length (μm) of adult worms according to different intestinal sites and days of infection

Site of infection		Day after infection				
		9	14	19	25	35
S-1	Mean length (S.D.)	2122 (336)	1647 (169)	1465 (136)	1166 (110)	N.D.
	No. examined	100	100	100	100	
S-2	Mean length (S.D.)	2108 (288)	1659 (152)	1331 (121)	1161 (99)	N.D.
	No. examined	100	100	100	79	
S-3	Mean length (S.D.)	1934 (260)	1552 (117)	1301 (112)	1164 (95)	
	No. examined	100	100	100	55	—
S-4	Mean length (S.D.)	1803 (548)	1414 (133)	1398 (159)	1169 (110)	1231 (206)
	No. examined	37	28	100	89	26
Cc	Mean length (S.D.)	N.D.	N.D.	1447 (146)	1247 (118)	1259 (95)
	No. examined			100	100	100
C-1	Mean length (S.D.)			1472 (140)	1283 (101)	1232 (94)
	No. examined	—	—	100	100	100
C-2	Mean length (S.D.)			N.D.	1274 (125)	1243 (102)
	No. examined	—	—		100	68

—, No worms recovered.
N.D., Not determined, as the number of worms was less than 10.

were infected with 3000 L3s (Group III) and 4 each were killed on days 9, 14, 19, 25 and 35 post-infection. The small intestine (divided into 4 equal parts and designated as S-1, S-2, S-3 and S-4), the caecum (designated as Cc), and the colon (divided into 2 equal parts as C-1 and C-2) were examined for adult worms using the Baermann apparatus, kept at 37 °C for 3 h. Further microscopical search was made to find adults adhering to the tissue fragments. The number of worms was recorded by location in the intestine. One hundred random worms, 25 each from 4 animals, recovered from different segments of intestine, or all worms if the number was fewer than 100, were fixed in AFA (alcohol, formalin and acetic acid fixative; 95% ethanol 25: formalin 5: glacial acetic acid 1: H_2O 20) and body length measured using a computerized Video Micro Meter (Model VM-30, Olympus, Tokyo). The number of embryonated eggs in the uterus was also counted.

Faecal culture and the numbers of L3s and free-living adults that developed

Using a new group of 5 rats infected with 3000 L3s (Group IV), Harada-Mori filter paper strip cultures of 400 mg of stool/animal on 2 separate filter paper strips were performed at 25 °C for 9 days at days 7, 11, 15, 17, 21, 25, 30 and 35 of infection. In order to

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Table 3. The mean number of embryonated eggs per parasitic female in the uterus according to different intestinal sites and days of infection

Site of infection		Day after infection				
		9	14	19	25	35
S-1	Mean no. of eggs (S.D.)	8·36 (2·60)	6·22 (2·81)	2·69 (2·41)	0·33 (0·73)	N.D.
	No. examined	100	100	100	100	
S-2	Mean no. of eggs (S.D.)	8·06 (2·27)	5·58 (2·23)	1·55 (1·96)	0·28 (0·78)	N.D.
	No. examined	100	100	100	85	
S-3	Mean no. of eggs (S.D.)	7·31 (2·51)	4·82 (2·02)	1·66 (2·09)	0·14 (0·51)	
	No. examined	100	100	100	58	—
S-4	Mean no. of eggs (S.D.)	5·34 (2·95)	2·89 (3·11)	3·44 (2·53)	0·18 (0·49)	0·63 (0·88)
	No. examined	38	28	100	90	27
Cc	Mean no. of eggs (S.D.)	N.D.	N.D.	1·83 (2·06)	1·66 (1·87)	1·36 (1·42)
	No. examined			100	100	100
C-1	Mean no. of eggs (S.D.)			3·62 (2·41)	2·02 (1·73)	1·26 (1·35)
	No. examined	—	—	100	100	100
C-2	Mean no. of eggs (S.D.)			N.D.	1·82 (1·42)	1·08 (1·34)
	No. examined	—	—		100	72

—, No worms recovered.
N.D., Not determined, as the number of worms was less than 10.

reduce any chance of bacterial contamination of culture water, the filter paper strip was transferred on the 4th day to a new culture tube, and the number of L3s in the old culture tube counted; on the 9th day of culture, the filter paper strip was submerged in water at 40 °C for 20 min, and L3s released from stool and filter paper were recovered in the sediment of the water (Arizono, 1976). The total number of L3s/400 mg of stool developed during the 9 days was determined. The number of excreted eggs and rhabditiform larvae/12 mg of stool was also counted on the same days of faecal culture. Another group of 6 rats infected with 8000 L3s (Group II), was studied with the same 9-day faecal culture for up to 88 days post-infection.

In order to quantify the number and ratio of free-living adult males and females developing in faecal cultures, 600 mg of stool/animal from Group IV rats were cultured on 3 separate filter paper strips for 4 days, and the number of adults was counted on the same days of infection as in the above 9-day faecal culture. In order to separate adult worms from stool cultures, the filter paper strip was submerged in hot water (40 °C), and the free-living parasites in the sediment were counted. After the 4-day culture, some adults had degenerated by the time of examination, but most of them were included as the sex identification was unmistakable.

Statistical analysis

Recovery rates of adult worms at different days and sites of infection (Table 1) were compared using χ^2 square tests. For comparison by site, the day of infection was first selected and 2×2 contingency tables were made by pairing any 2 sites. Similarly, in each site of infection, the recovery rates were compared between any 2 days of infection using 2×2 contingency tables. In no case of the present multiple comparisons, days or sites of infection were combined. For the computation, adults from 4 animals were totalled by site and day of infection, and the total numbers were used instead of the averages presented in the table. The average length of adult worms and the number of embryonated eggs in the uterus (Tables 2 and 3) were analysed by day or site of infection using analysis of variance. Comparisons between any 2 different days or sites were made with Scheffé's multiple comparison. Differences were considered significant at $P < 0{\cdot}05$. When one kind of comparative procedure, such as χ^2-test, was repeated with different sets of data to draw a conclusion, the largest P value obtained is quoted in the text.

RESULTS

The number of eggs plus larvae in 12 mg of faeces is shown in Fig. 1. In Group I, the highest count was 1644 at day 8 of infection. The number dropped sharply and came to a sudden halt at day 20, making a plateau which lasted for about 10 days. In the heavier infection of Group II, a longer lasting second peak, the existence being verified by the depression in egg count at day 18 which was significantly lower than the count at day 21 (t-test, $P < 0{\cdot}01$) or day 23 ($P < 0{\cdot}001$), was observable with a maximum count at around day 25. After day 14, many eggs were apparently dead and degenerated, but such eggs were few around day 25 post-infection.

The distribution of parasitic adults in the intestine varied significantly during the infection (Table 1). When the recovery rates were compared by site on different days post-infection, on days 9 and 14, higher rates of recovery were obtained in each of S-

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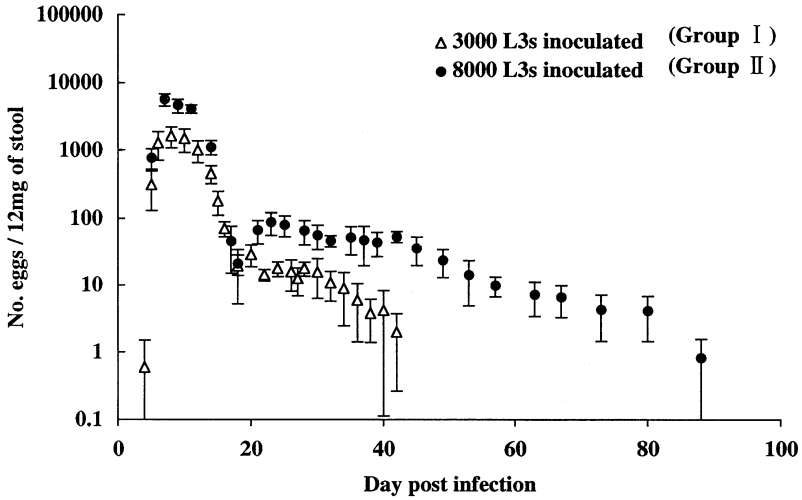

Fig. 1. The number of *Strongyloides ratti* eggs excreted in faeces in 2 groups of rats inoculated either with 3000 (Group I) or 8000 (Group II) infective larvae. Results are presented as average of 5 (Group I) or 6 (Group II) animals ± S.D.

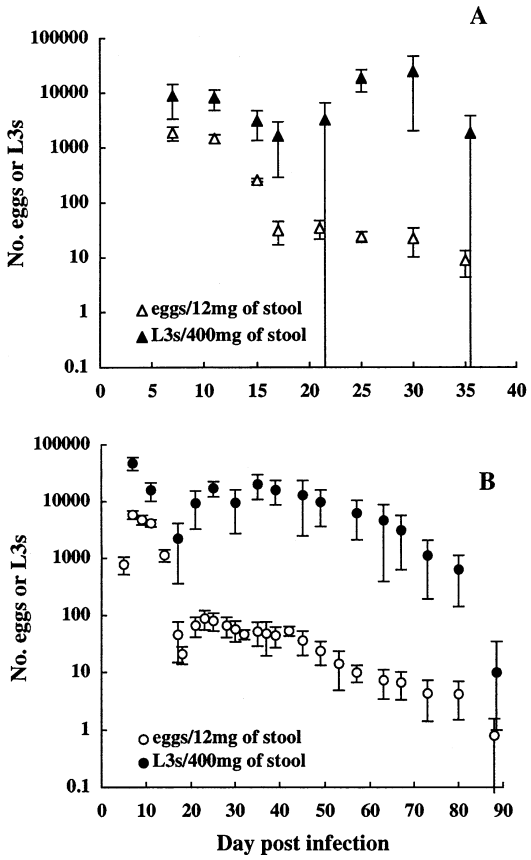

Fig. 2. Change in the numbers of *Strongyloides ratti* eggs excreted, and infective larvae (L3s) developed in 9-day faecal cultures in 2 groups of rats inoculated with 3000 L3s (A) or 8000 L3s (B). Results are presented as (A) average of 5 animals (Group IV) ± S.D.; (B) average of 6 animals (Group II) ± S.D. Note that the scales for day post-infection are different between A and B.

1 and S-2 of the small intestine than in each of S-3, S-4, Cc, C-1 and C-2 (χ^2-test, $P < 0{\cdot}001$ in all comparisons). A big shift in worm distribution was observed on day 19 when the recovery rate was the highest in the caecum (χ^2-test, $P < 0{\cdot}001$). On days 25 and 35, each of the colon (C-1) and the caecum had much higher rates than each of S-1 through S-4 and C-2 (χ^2-test, $P < 0{\cdot}001$). Compared by day of infection in each site, almost the same results were obtained. These findings indicate clearly the shift in the distribution from the small to large intestines.

Adult body lengths (Table 2) became shorter with the passage of time in each of S-1, S-2 and S-3, where average lengths were significantly different in any combination of 2 neighbouring days (Scheffé, $P < 0{\cdot}001$). A similar trend of shortening with time was noticed in C-1 (Scheffé, $P < 0{\cdot}05$). When analysed by site of infection on days 9 and 14, the proximal part of the small intestine (each of S-1 and S-2) harboured longer worms than the distal part (each of S-3 and S-4) (Scheffé, $P < 0{\cdot}05$). However, on day 25, worms in each of the caecum, C-1 and C-2 were significantly longer than worms in any site in the small intestine (Scheffé, $P < 0{\cdot}01$). The number of embryonated eggs in the uterus (Table 3) showed a decrease with time in each of S-1, S-2 and S-3. The averages were significantly different in any combination of 2 neighbouring days (Scheffé, $P < 0{\cdot}01$). Analysed by site of infection, worms in S-4 had fewer eggs than those in the proximal part (each of S-1 through S-3) on days 9 and 14 (Scheffé, $P < 0{\cdot}05$). At days 25 and 35, however, the average number of embryonated eggs was significantly more in worms from each of the caecum, C-1 and C-2 than in worms from any site in the small intestine (Scheffé, $P < 0{\cdot}001$). At day 25, adults in the caecum and the colon had 7·6 times more eggs in the uterus (the average of 300 worms: 1·83) than those in the small intestine (the average of 333 worms: 0·24) (t-test, $P < 0{\cdot}001$).

The production of L3s, either by direct development from rhabditiform larvae or from free-living adults, was measured by counting the total

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Table 4. Total output of eggs and L3s during the first peak and the second peak or plateau of egg excretion*

	Total egg output	Total L3 output
3000 L3s inoculated (Group IV)		
1st peak (Days 5–18 of infection)	13023	76315
Plateau† (Days 19–35 of infection)	377	129502
8000 L3s inoculated (Group II)		
1st peak (Days 5–18 of infection)	36163	256434
2nd peak (Days 19–88 of infection)	1934	541464

* Average output of 5 (in Group IV) or 6 (in Group II) animals was accumulated daily for the period of time indicated as Days of infection.
† The second peak was not apparent.

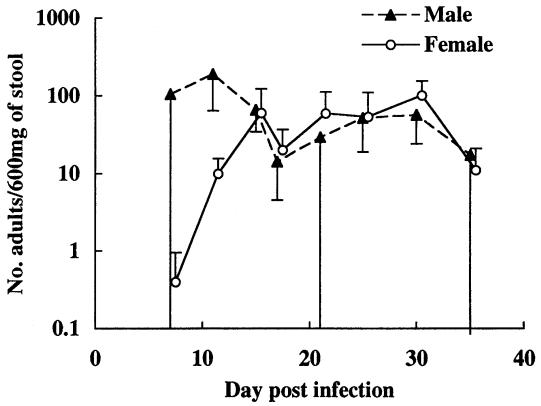

Fig. 3. Change in the number of free-living adults that developed in 4-day faecal cultures. Results are presented as the average of 5 animals (Group IV); +S.D. for females and −S.D. for males. For a technical reason, only 4 animals were used on days 30 and 35.

number of L3s which developed in 9-day-old faecal culture (Fig. 2A, B). Despite the fact that egg counts dropped sharply after day 10 of infection, the total number of L3s remained at a remarkably high level, even at day 30 post-infection in Group IV (24037 L3s), or at day 57 in Group II (6242 L3s), though a clear depression in L3 production was noticed at day 17 (2234 L3s) in this case. The total numbers of eggs excreted and L3s developing during the first peak and the second peak or plateau were estimated by accumulating daily outputs in Table 4, in which the first peak includes days 5 through 18, based on the result that day 18 had a significantly lower egg count than the following days in the Group II experiment (Fig. 1). In the case of the Group IV experiment in Table 4, the day 18 post-infection divided the first peak and the following plateau, that is, the second peak was not apparent in Group IV. Data for days not examined were estimated by interpolation, and particularly for days 5 and 6, daily output was regarded as 20% and 80% of day 7 value, respectively, the percentages being estimated from our preliminary study using the same *S. ratti*–rat model. The egg excretion during the second peak or the plateau was about one twentieth to one thirty-fifth of the first peak; the L3 output, however, was about twice that of the first peak. The number of free-living adults obtained in 4-day culture of stool at various days after infection revealed that very few females developed during the first peak of egg excretion and the sex ratio became approximately 1 after day 15 (Fig. 3), when rapid expulsion of parasitic adults from the small intestine was expected to be occurring.

DISCUSSION

A high peak of egg excretion was observed at day 7 or 8 of infection when the majority of adults were in the first half of the small intestine. This is the common observation reported by many workers (Moqbel & Denham, 1977; Korenaga *et al.* 1983). In addition, this study established the presence of a second peak of egg excretion with this Nagoya strain of *S. ratti*, though in Groups I and IV a plateau rather than a second peak was seen. The existence of the second peak was noticed by some researchers, but was ignored, probably because the magnitude was relatively small. In some experiments, and also under natural conditions, the number of L3s inoculated might not be sufficient to produce a recognizable second peak. The present study revealed, during the second peak or the plateau (1) a coincidental increase in the number of adults in the caecum and the colon and (2) a larger body length of the adults and more embryonated eggs in their uteri, compared with concurrently parasitic adults in the small intestine. These results would suggest that the second peak or the plateau was produced by adults in the caecum and the colon. It is possible that, once adults are dislodged from the small intestine, they resettle in the caecum and the colon, recover from damage received in the previous parasitic site and then have greater fecundity (Moqbel, McLaren & Wakelin, 1980). However, no direct evidence of regrowth was obtained in this study. The possibility that there were two populations of worms, one which matured early and inhabited the small intestine and the other which matured late and inhabited the

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caecum or the colon, can be excluded, because out of 2334·3 worms recovered from the small intestine at day 9, only 180·0 were less than 1600 μm in length, whereas, at day 25, there were 408·2 worms of the same size category in the caecum and the colon. The present result would imply that there are different mechanisms of worm expulsion between the small and the large intestines. We could not study the relative importance of the caecum and the colon.

Despite the fact that the first peak of egg excretion decreased sharply, the total L3 output, as measured by 9-day faecal cultures, was maintained for a month or two. Meanwhile, a separate experiment with 4-day culture of stool at different days of infection revealed that free-living females were very few at the time of the first peak; the number of females increased gradually bringing the sex ratio to 1 after day 15 of infection. These results indicate that most eggs in the first peak developed directly to L3s (homogonic L3s), whereas the eggs in the second peak tended to develop into the free-living cycle in which indirect heterogonic L3s were produced. The change of direction in larval development, from homogonic to heterogonic, was described in detail by Viney (1996) with isofemale lines of *S. ratti*. Possible reasons for the shift would be; low larval population density or sufficient faeces during the time of second peak (Arizono, 1976; Shiwaku *et al*. 1988), acquired host immunity which induced the free-living adult cycle (Gemmill, Viney & Read, 1997), higher concentrations of unsaturated fatty acids in the small intestine than in the large intestine (Minematsu *et al*. 1989) and involvement of genetically different isofemale lines (Viney, Matthews & Walliker, 1992). As the total number of L3s produced during the second peak or plateau was twice that of the first peak, this small peak or plateau seems to have a potentially important role in the transmission of *S. ratti*. Moreover, considering that free-living males and females are the only stage to exchange genes in this life-cycle (Viney, Matthews & Walliker, 1993; Viney, 1994), the shift and the maintenance of the heterogonic cycle might constitute a survival strategy of *S. ratti* enabling needed genetic modification. However, as external environmental conditions also can change the direction of larval development, the importance of parasitism in the caecum and the colon has to be further clarified. Additionally, our results were obtained using rats infected with a very high count of L3s. The relevance of the study to natural infections remains to be elucidated.

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