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The Mode of Active Protection against *Hymenolepis nana* Reinfection in Mice Inoculated with Different Doses of Shell-Free Eggs

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Introduction

Protective immunity against reinfection with *Hymenolepis nana* can be induced in mice within 1–2 days following an initial inoculation of eggs (Hearin, 1941; Heyneman, 1962), but the precise mechanism of immunity against *H. nana* is unknown (Gemmell and Macnamara, 1972; Larsh, 1951; Larsh and Weatherly, 1975; Weinmann, 1966). To our knowledge whether the presence of even single egg-derived tapeworm infection is sufficient to make the host complete immune is not proved (Heyneman, 1963).

As the index of protection in *H. nana* infection, the well known evidence that no cysticercoid recovers from subsequent inoculation of eggs has been used (Hunninen, 1935). However, the index is an oversimplification of the events which occur in the intestine, since it provides no information as to whether the lethal effect was directed against the egg, the oncosphere in the intestinal lumen, the oncosphere in the villus, or the early post-oncospheral phase in the villus (Gemmell and Macnamara, 1972).

The aim of the present study was therefore to determine whether mice harboring different number of egg-derived tapeworms showed difference in responsiveness to *H. nana* reinfection, and to identify the stage at which protection occurred in these immune hosts.

Materials and Methods

Eggs of *H. nana*, free of debris, were teased from gravid proglottids, suspended in 0.9% NaCl solution and stored at 4 C for a few days before use. Prior to inoculation the shells of more than 95% of eggs were removed by cracking them with glass beads (Berntzen and Voge, 1965). Different doses of shell-free eggs varying 5–2,000 were resuspended in 0.1 ml of 0.9% NaCl solution.

The mice used in this study were random-bred albino dd mice of both sexes raised in our laboratory. Maintenance of uninfected mice has been described previously (Ito, 1975). Uninfected mice of 5–6 weeks old were initially inoculated with 5–2,000 shell-free eggs per mouse (day 0). All the mice were housed in wire-bottomed plastic cages, 7–12 mice per cage. Food and water were available *ad libitum*. The cage was renewed daily throughout experiments. Details of the experimental design are given in the description of each experiment.

All the mice were killed by breaking their necks and the recovery of tapeworms from initial egg inoculation was counted (Ito, 1975). The maturity of the tapeworm was determined by examining the presence or absence of gravid proglottids by microscopic observation under ×100 magnification. After removal of the tapeworms, the intestine wall was stored in fresh tap water at 4 C overnight. Then the intestine was examined microscopically for the presence of cysticercoids under ×40 magnification. In Exps. 1 and 2, the presence or absence of earlier stage larvae other than cysticercoids was also examined by observing the oncospheral hooks under ×400 magnifica-

tion.

Experimental Design and Results

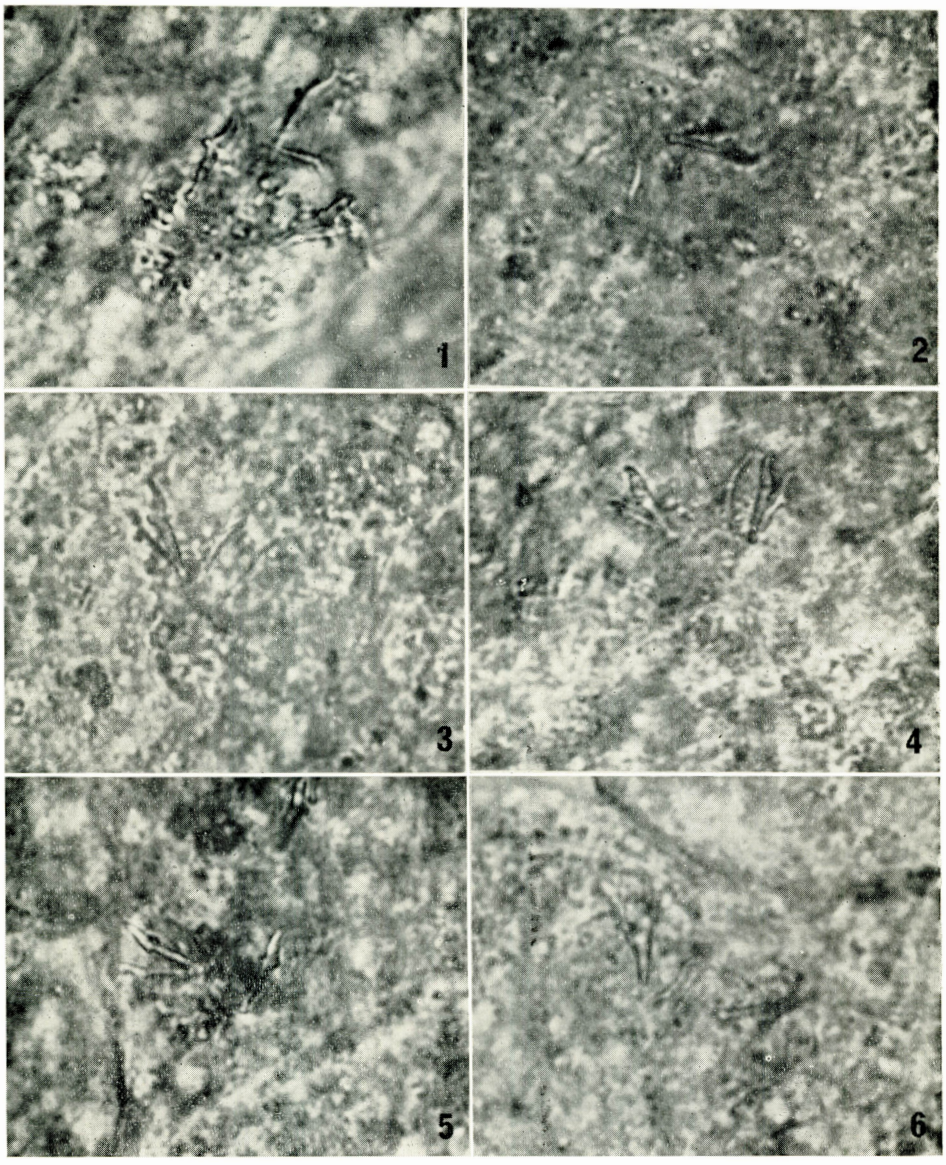
Exp. 1. It was examined to determine whether protective immunity against *H. nana* reinfection in mice was acquired by single egg-derived tapeworm infection and to identify the stage of *H. nana* and the site at which protection achieved. A total of 154 mice out of 164 mice was initially inoculated with different doses of shell-free eggs varying 5–2,000 per mouse on day 0, challenged with 2,000 shell-free eggs on day 10 and killed on day 14. Each of the other 10 mice of the 164 mice was used as the control and initially inoculated with 2,000 shell-free eggs on day 0 and killed on day 4. Recovery of tapeworms from initial egg inoculation and that of cysticercoids from challenge was counted in the 154 mice and recovery of cysticercoids from initial inoculation was counted in the 10 control mice killed on day 4. The results are shown in Table 1; 143 mice out of the 154 mice harbored 1–464 tapeworms and either the remaining 11 (*, in Table 1) or the 10 controls did no tapeworms.

When the presence or absence of cysticercoids in the villi 4 days post challenge was examined, almost all the mice harboring one or more tapeworms (137/143 mice) were not infected with any cysticercoids in the villi. The other 6 mice out of the 143 mice were found to be infected with significantly fewer 1–189 cysticercoids than the 10 controls harboring 413.5±120.16 (S.D.) ones. However, when no tapeworm was recovered from the 11 mice out of the 69 mice inoculated with less than 10 shell-free eggs, the mice were found infected harboring 420.1±103.78 (S.D.) cysticercoids and possessed no immunity. Further, there was no significant difference in number of *H. nana* by counting tapeworms on day 14 and cysticercoids on

Table 1 Recovery of initial egg-derived tapeworms in the intestinal lumen and the presence or absence of secondary egg-derived cysticercoids or oncospheres in the villi of the mice harboring the tapeworms

Initial doses of shell-free eggs day 0	No. of mice harboring tapeworms day 14 / No. of mice inoculated day 0	No. of tapeworms in infected mice			No. of mice harboring		
		Mean	S.D.	(range)	None	Onco.	Cyst.
7– 8	8/ 8	2.0	1.60	(1– 5)	5	NT	3
	11/11	2.3	1.49	(1– 5)	10	NT	1
	9/10	1.7	1.12	(1– 4)	9	NT	0(1)*
	8/ 8	1.4	0.53	(1– 2)	8	NT	0
16	10/10	9.4	3.50	(3– 13)	10	NT	0
	10/10	5.7	3.20	(2– 11)	10	NT	0
5– 9	6/10	2.1	0.97	(1– 4)	2	4	0(4)*
	9/12	2.7	1.32	(1– 5)	1	8	0(3)*
	7/10	2.5	1.14	(1– 4)	0	7	0(3)*
10–12	8/ 8	5.0	1.89	(2– 7)	5	3	0
15–20	12/12	4.5	4.07	(1– 12)	7	4	1
30–34	7/ 7	14.3	3.86	(9– 21)	2	5	0
200	8/ 8	73.8	20.08	(49–108)	4	3	1
500	10/10	171.2	48.84	(108–245)	2	8	0
2,000	10/10	370.0	77.75	(298–464)	0	10	0
2,000(control)	10†/10	413.5††	120.16††	(301–517)††	0	0	10

NT: not tested, Onco.: oncospheres, Cyst.: cysticercoids, ( )*: No. of mice harboring cysticercoids but no tapeworms, †: No. of mice harboring cysticercoids killed on day 4. ††: No. of cysticercoids recovered on day 4.



Explanation of Plate

Photomicrographs of the oncospheres found in the intestinal villi 4 days post secondary egg inoculation (day 14).

Patterns of the hooks and relative sizes of the worms to the hooks in these figures (Figs. 1-6) demonstrate that the worm is the oncosphere derived from secondary egg inoculation and no reorganization of the oncosphere has occurred for 4 days.

day 4.

When the intestines harboring no cysticercoids were carefully observed under ×400 magnification, a few oncospheres was found in most of them. It was very hard to find out single oncosphere from one intestine and therefore the results of this observation were qualitative; when one or more oncospheres were found in the intestine, the mouse was judged to be harboring oncos-

pheres. Figs. 1–6 illustrate instances of the presence of such oncospheres in the intestine walls of 6 different mice. Either the mouse harboring 464 tapeworms or that with only one tapeworm did similarly harbor the oncospheres in the villi on day 14.

Exp. 2. This experiment was done to determine whether the oncospheres found on day 14 were derived from the challenge on day 10 or from the initial inoculation on day 0. Thirty mice were divided into three groups of 10 animals each. The mice of groups A and B were inoculated with 500 shell-free eggs per mouse on day 0. Mice of group A were killed on day 14, while those of group B were killed on day 10. Mice of group C were untreated and killed on day 14. The result is shown in Table 2. The mice of group A were found infected with 157.4±46.53 (S.D.) mature tapeworms, whereas those of group B with 138.1±51.26 immature ones. Neither the oncospheres nor the cysticercoids were observed in the villi of the 10 mice of group B and 8 out of the 10 mice of group A at all, whereas the other two of group A harbored more than 50 oncospheres, but no cysticercoids. No mice of group C harbored any stages of *H. nana*.

Exp. 3. This experiment was done to see if the oncospheres found in the villi on day 14 could develop to cysticercoids or tapeworms. Each of 20 mice was inoculated with 500 shell-free eggs on day 0 and challenged with 2,000 shell-free eggs on day 10. Ten of the 20 mice were killed at random on day 14, the other 10 killed on day 21, and the number of both tapeworms in the lumen and cysticercoids in the villi was compared between the two groups. The mean number of tapeworms recovered from the mice killed on day 14 was 167.8±36.28 (S.D.) and that from another group of mice killed on day 21 was 171.2±48.84. There was no significant difference in number of tapeworms recovered between these two groups. In addition none of the mice of the two groups harbored any cysticercoids.

Discussion

The present results show that protective immunity against *H. nana* reinfection in the mouse is acquired by the presence of single egg-derived tapeworm infection, *i.e.*, even one tapeworm established by the exposure of a very small number of shell-free eggs, such as less than 10 is sufficient to induce strong protective immunity. Although Berntzen and Voge (1965) found that the rate of infection is much higher by the use of shell-free eggs than by intact eggs, a number of intact eggs was inoculated to the mouse for induction of protective immunity against *H. nana* by many workers. Therefore whether most of eggs in the lumen which fail to penetrate the intestine wall have any role as the immunogens has been ambiguous. However, it is strongly suggested from the present results that eggs in the lumen do not act as the immunogens and is confirmed that the oncosphere in the villus is strongly im-

Table 2 Developmental stages of *Hymenolepis nana* in mice on day 10 or on day 14 when the mice were inoculated with 500 shell-free eggs once on day 0

Group	Shell-free eggs inoculated per mouse	Day of necropsy	No. of mice harboring Tapeworm (maturity)	No. of mice inoculated Onco.	Cyst.
A	500	day 14	10/10(mature)	2/10	0/10
B	500	day 10	10/10(immature)	0/10	0/10
C	0	day 14	0/10	0/10	0/10

Each group consisted of 10 mice.
Onco. : oncospheres, Cyst. : cysticercoids.

munogenic (Hearin, 1941 ; Heyneman, 1963).

Provided that the index of protection was whether cysticercoid derived from subsequent egg inoculation was formed in the villi 4 days post challenge, the protection induced by one or more egg-derived tapeworm infection was complete as previously suggested (Bailey, 1951 ; Hearin, 1941 ; Heyneman, 1961 ; Larsh, 1951). In the majority of the immune hosts harboring more or less tapeworms, however, oncospheres were found in the villi, although it was very hard to find out the oncospheres. There was no evidence that the size of initial infection affected the number of oncospheres found in the villi ; in any size of initial infection, a very small number of oncospheres derived from challenge was found 4 days post challenge. These results strongly suggest that the site of protection is both the lumen and the villi and the stage of *H. nana* is the oncosphere.

Weinmann (1966) found that the influence of mucosal extract of immune mice on immature worms (but not the oncospheres) was striking *in vitro* and suggested that the site of protection was the lumen. Nevertheless, some oncospheres did invade the villi. Therefore it may be more probable to consider that host immune response is directed against the oncosphere ; the first is to inhibit the invasion of oncospheres into the villi and the second to inhibit differentiation of the oncospheres to cysticercoids in the villi, even if they have evaded the first host immune response. Furukawa (1974) reported a method of lymphoid cell adherence on the oncosphere-antibody complex for demonstrating antibodies against *H. nana*, although it was based on Ito's (1975) original work that immune serum contained antibodies against the oncosphere and induced oncospheral agglutination as the antigen-antibody complex. These reports supported the suggestion that the oncosphere is strongly immunogenic (Heyneman, 1962 ; Leonard and Leonard, 1941 ; Weinmann, 1966) and agreed with the present results.

In two intestines out of the 10 mice harboring mature tapeworms, more than 50 oncospheres were found on day 14. This unexpected finding was never observed in the mice harboring immature tapeworms. Therefore this may be due to the autoreinfection by the eggs emerged from the mature tapeworms (Heyneman, 1961).

There is an experimental evidence that passive immunity is transferred by immune mouse serum (Ito, in preparation) : in mice given immune serum, the mode of protection was slightly different from that in actively immunized mice. The oncospheres invaded the villi but failed to differentiate to cysticercoids. There was no significant difference in number of the oncospheres found in the villi of mice given immune serum and of untreated control mice. The lethal effect was demonstrated when the serum was given to the mouse within one day post egg inoculation, although the oncospheres invaded the intestinal villi within 4 hr post egg inoculation (Miyata, 1944). The discrepancy between the mode of protection in actively immunized mice and that in passively immunized ones may suggest that the intestinal mucosa of actively immunized mice serves as important barrier against oncosphere invasion (Bailey, 1951 ; Leonard and Leonard, 1941 ; Musoke and Williams, 1975 ; Weinmann, 1966).

These evidences may support the following hypothesis that the host immune response can be induced when one oncosphere originating from initial egg inoculation develops into cysticercoid stage in the villus of the small intestine and directly associated with the development of the oncospheres derived from subsequent inoculations and exists both in the lumen and in the villi.

Summary

By the use of a small number of shell-free eggs of *H. nana* it was found that there was no threshold of protection against *H. nana* reinfection. Whenever one or more egg-derived tapeworm infection succeeded in the mouse host, the host became complete immune : The presence of single tapeworm

derived from a very small number of shell-free eggs such as less than 10 was sufficient to make the host immune. No cysticercoid derived from secondary inoculation of eggs were formed in the immune host. However, most of the immune hosts harbored a few oncospheres in the villi. The oncosphere was not derived from initial inoculation but from secondary inoculation and did never develop to cysticercoid or tapeworm. Therefore it seems highly probable to consider that the host immune response can be induced when single oncosphere originating from initial egg inoculation develops into cysticercoid stage in the villus of the small intestine and this immune response is directly associated with the development of oncospheres which are derived from subsequent inoculations and exists both in the lumen and in the villi.

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小形条虫の脱殻虫卵投与マウスの再感染防御

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種々の卵数（5-2,000個）の小形条虫脱殻虫卵（以下虫卵と略す）を経口投与したマウスにおける成条虫寄生数と再感染に対する抵抗性を虫卵初投与後10日目に2,000個の虫卵を再投与，その4日後に剖検する方法で調べ次の結果を得た．

1)　154匹中143匹のマウスから投与虫卵数に応じ，少なくとも1隻以上（1-464隻）の成虫が検出された．成虫寄生マウス143匹中137匹からは全く擬嚢尾虫は検出されなかつたが残り6匹からは1-189隻の擬嚢尾虫が検出された．一方成虫寄生がみられなかつたマウス11匹からは420.1±103.78（S.D.）の擬嚢尾虫が検出された．更に対照として2000個の虫卵初投与後4日目に剖検した10匹からも413.5±120.16の擬嚢尾虫が検出され両者間に擬嚢尾虫寄生数に関して有意差は認められなかつた．

2)　擬嚢尾虫の検出されなかつたマウス腸管を詳しく観察したところ大半のマウス小腸絨毛内から六鉤幼虫が検出された．しかし成虫寄生数の多少に関りなく検出された幼虫数は非常に少なかつた．

3)　この六鉤幼虫は再投与虫卵由来であり擬嚢尾虫あるいは成条虫に発育分化することはなかつた．

以上の結果は宿主の再感染防御免疫は1隻の六鉤幼虫が宿主小腸絨毛内で擬嚢尾虫に発育分化することによつて成立すること，2度目の六鉤幼虫の絨毛内侵入をかなり強く阻害するが幼虫の侵入を完全に阻止するものではないことを示すものである．