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Protective Immunity in Mice Subcutaneously Inoculated at an Early Age with *Hymenolepis nana* Eggs

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Although a single *oral* infection of mice with eggs of *Hymenolepis nana* stimulates rapid development of a strong immunity to reinfection, there have been no studies on induction of protective immunity by *Parenteral* inoculation with eggs. It was established that shell-removed eggs of *H. nana* would develop in the usual manner to the cysticercoid stage within 5 days when injected subcutaneously or intramuscularly into previously uninfected mice (Di Conza, 1968). The majority of cysticercoids which developed subcutaneously in normal mice survived 3 weeks (Di Conza, 1968), but no subcutaneous development of cysticercoid larvae was observed when cracked eggs were injected subcutaneously into mice as late as 21 days after previous *oral* egg infection (Furukawa, 1971). The immediate objective of the work reported here was to examine whether or not mice would induce protective immunity against the subsequent oral challenge with eggs of *H. nana* if subcutaneously inoculated with living eggs resulting in the subcutaneous development of cysticercoids. Experiments using neonatally thymectomized mice have also been carried out in induction of the protective immunity.

Materials and Methods

The animals used for experiments were ICR mice born of uninfected mother. Some of the mice were subjected to neonatal thymectomy during the first 18 hours after birth by the method as described previously (Okamoto, 1968). Eggs of *H. nana* were teased from gravid proglottides and washed several times with sterile physiological saline. Their outer membranes were cracked and removed by stirring with glass beads for approximately 5 minutes according to the method of Berntzen and Voge (1965). Saline rinsed "cracked" eggs were then suspended in physiological saline with penicillin added to a concentration of 4,000 U/ml and left for 30 minutes. Following centrifugation (3,000 rpm), as much as possible of the supernatant saline was removed. The sediment was resuspended in physiological saline. The eggs were then injected subcutaneously into 1- and 4-week-old mice using a tuberculin syringe (size: 0.25 ml). The dose was 5,000 and 50,000 eggs in a volume of 0.05 ml. The inoculation site, especially the needle puncture in the region of the back, was carefully sealed with nail enamel and dried to prevent oral contamination. Two different types of eggs, living and killed, were employed in the subcutaneous inoculation. Killed eggs were prepared by lyophilizing cracked eggs in a test tube for 5 hours before suspending them in the sterile physiological saline. The inoculation procedure was then similar to that used with living eggs.

To evaluate the degree of immunity resulting from one subcutaneous inoculation, mice were challenged by oral exposure to 2,000 cracked eggs in 0.05 ml of saline. The eggs were administered using a tuberculin syringe fitted with a blunted needle either 1 or 3 weeks after subcutaneous inoculation. The method of Hunninen (1935) was used in examining small intestine for cysticercoids, i. e., the mice were killed 4 days after challenge and the intestinal villi were examined for cysticercoids derived from the oral egg challenge. The protection afforded by the subcutaneous inoculation for each mouse was determined by the absence of the intestinal cysticercoids from the oral challenge.

Results

Results are summarized in Table 1 with the number of mice in each of the following categories based on cysticercoid counts: 0, 1-9, 10-99, >100. The age of the mice at the time of subcutaneous inoculation was either 1 week or 4 weeks. In all the mice inoculated subcutaneously with 5,000 eggs at 1 week of age and challenged orally 1 week later, no cysticercoids were found which developed in the intestinal villi. Whereas, all the mice inoculated with the same egg dose at 1 week of age and challenged orally 3 weeks later possessed the intestinal cysticercoids derived from the challenge. In the inoculation experiments with 50,000 eggs, 13 of the 15 mice inoculated at 1 week of age were negative for intestinal cysticercoids from the challenge, even when the mice were challenged orally 3 weeks later. In contrast, almost all of the mice gave evidence of challenge parasite development in the villi when they were subcutaneously inoculated at 4 weeks of age, in spite of the same intervals from inoculation to challenge as those of experiments with low-egg dose (5,000 eggs). All of the 8 mice were positive for the intestinal cysticercoids when they were challenged 1 week after inoculation. Six of 9 mice also harbored intestinal cysticercoids when they were challenged 3 weeks after inoculation. These results not only indicate

Table 1 Protective immunity of mice inoculated subcutaneously with eggs of *Hymenolepis nana*

Subcutaneous inoculation with	Age at inoculation (weeks)	Age at challenge (weeks)	No. of mice examined	No. of mice with following cysticercoid counts				No. infected / No. challenged
				0	1-9	10-99	>100	
Controls	—	2	3	0	1	1	1	3/ 3
	—	4	38	0	0	0	38	38/38
Nonthymectomized mice								
5,000 shell-removed eggs	1	2	12	12	0	0	0	0/12
	1	4	17	0	5	7	5	17/17
50,000 shell-removed eggs	1	4	15	13	0	2	0	2/15
	4	5	8	0	1	5	2	8/ 8
	4	7	9	3	1	3	2	6/ 9
50,000 lyophilized eggs	1	4	9	0	0	0	9	9/ 9
Neonatally thymectomized mice								
5,000 shell-removed eggs	1	2	11	1	7	3	0	10/11
50,000 shell-removed eggs	1	4	8	0	0	3	5	8/ 8

that prior subcutaneous inoculation with eggs of *H. nana* to a great extent elicites protective immunity to oral infection with eggs, but also that the age of the mouse at the time of inoculation influences its ability to evoke protective immunity to *H. nana* in the intestinal villi.

It was of interest to see if killed eggs were effective in the induction of protective immunity. Nine mice inoculated subcutaneously with 50,000 lyophilized eggs at 1 week of age and challenged orally 3 weeks later were all positive for the intestinal cysticercoids from the challenge. All of the mice harbored more than 100 cysticercoids. Almost all of the mice thymectomized at birth suffered oral infection when they were inoculated subcutaneously with either 5,000 or 50,000 living eggs at 1 week of age. All the mice, including nursing mothers, used in the present experiments were completely free of the lumen-dwelling adult worms from subcutaneous inoculation and/or from oral contamination.

Discussion

When *Hymenolepis nana* eggs were injected subcutaneously into mice, the parasites appeared to remain localized and developed to cysticercoids. From the data presented, it is obvious that the subcutaneous development of *H. nana* cysticercoids from living shell-removed eggs was immunogenic to the subsequent oral egg infection. In mice inoculated with 5,000 living eggs at 1 week of age, all the mice were resistant against the subsequent oral challenge with eggs, when the interval between inoculation and challenge was 1 week, but none of the mice were resistant when it was 3 weeks. Whereas, in mice inoculated with high-dose eggs (50,000 living eggs) at 1 week of age, almost all of the mice were resistant even when the interval was 3 weeks.

Insofar as migration of lymphocytes into the mouse small intestine are concerned, several answers to this question are already available. Parrott and Ferguson (1974) reported the results of extensive research on the migratory behaviour of lymphocytes to lamina propria or Peyer's patches of mouse small intestine. They indicated that tritiated thymidine labelled cells from mesenteric lymph nodes of donor mice infected subcutaneously with a thousand *Nippostrongylus braziliensis* larvae were found in Peyer's patch tissue and lamina propria of both grafts of feotal small intestine under kidney capsule and normal small intestine, but the labelled lymphocytes from oxazolone-primed lymph nodes did not migrate to the villi. Direct evidence for the thymic origin of most lymphocytes in developing Peyer's patches of newborn mice has also been obtained by isotopic marker experiments (Joel *et al.*, 1971). They showed that thymic migration begins earlier and in mice beyond the neonatal stage only a few thymocytes which migrated from the thymus to Peyer's patches were found in the intestine. This fact may be partly due to the large number of lymphocytes already present in the gut-associated lymphoid tissue at that age. These studies raise the possibility that, in immature mice at least, lymphocytes migrate from lymph nodes and thymus to Peyer's patches and/or lamina propria. The data obtained here also showed that, if mice were 4 weeks old when inoculated subcutaneously with 50,000 *H. nana* eggs, a high-level of protection did not develop within 3 weeks after inoculation. This result indicates an inability of mice over 4 weeks of age to become immune to *H. nana* oral infection by the parenteral inoculation of *H. nana* eggs, while mice below 1 week of age had an ability to become immune.

It would appear that the concept of local immunity in the intestinal tract, which has recently been investigated and described in relationship to antibody (mainly secretory Ig A), must be expanded to include also cell-mediated immunity. In assessing the mechanisms of immunity against *H. nana* not only should the location of the immunity ; i. e., intestinal villi (oral) versus systemic (parenteral) be considered, but also the relative

roles of humoral and cellular immunity. The experiments with neonatally thymectomized mice showed that the intestinal defence mechanism evoked by the parenteral inoculation with *H. nana* eggs is thymus-dependent, just as the protection elicited by the previous oral administration of eggs is (Okamoto, 1968).

Astafiev (1966) reported the possibility of migration of *H. nana* from intestinal tract and finding cysticercoids in the liver, spleen, lungs and mesenteric lymph nodes. When *H. nana* eggs were injected subcutaneously, the cysticercoids appeared to remain localized (Furukawa, 1971). Di Conza (1970) stated that, when a light immunizing infection was given to mice subcutaneously and these mice were later challenged with eggs orally, few larvae succeeded in establishing in the intestinal villi. In the present study, we indicated that some of the mice injected subcutaneously with living eggs were resistant against the subsequent oral egg challenge. The experiment reported here did not include a group of inoculated but not challenged mice. We realized that a subcutaneous inoculation of 5,000 to 50,000 living "cracked" eggs might lead to a few adults in the intestinal lumen. This could occur either by mice licking at the injection site after removal of the needle and leakage of some of the eggs or by direct migration of some larvae from the injection site. However, all the mice, including nursing mothers, were completely free of the lumen-dwelling adult worms from oral contamination and/or subcutaneous inoculation at autopsy. This indicated us that the inclusion of a inoculated but not challenge group was not necessary in evaluating the effect of subcutaneous development of cysticercoids on acquired immunity.

Previous work by Okamoto *et al.* (1974) has indicated that the upper limit of the period of an unresponssive state varies in different strains of mice but, in ICR mice, not extends far beyond about 1 week of age. There is interest in around the time of 1 week in age of mice as a critical point responsible for the development of immunological facilities. The result, presented in this paper, shows that the earlier the mice were inoculated subcutaneously with eggs, the higher the acquired immunity in the intestinal villi against *H. nana* oral infection. Although highly speculative, one could consider the hypothesis that the locality of immune mechanisms in the intestinal tract is ontogenitically built up; i. e., such locality does not fully develop yet in early life of mice during which lymphocytes migrate into gut-associated lymphoid tissue. Further works along this hypothesis with more suitable antigens will be necessary.

Summary

Shell-removed eggs of *Hymenolepis nana* were subcutaneously inoculated into 1- and 4-week-old mice. These mice were orally challenged with 2,000 eggs 1 week or 3 weeks after the inoculation. The degree of protection against oral challenge was assessed by examination for the absence of intestinal cysticercoids 4 days after the challenge. In mice inoculated with 5,000 eggs at 1 week of age, all the mice were resistant against oral challenge when the interval between inoculation and challenge was 1 week, but none of the mice were resistant when it was 3 weeks. In mice inoculated with 50,000 eggs at 1 week of age, almost all of the mice were resistant even when the interval was 3 weeks. If mice were 4 weeks old when inoculated with 50,000 eggs, a marked degree of protective immunity did not develop in their intestinal villi. The results also indicate that the defence mechanism evoked by the parenteral inoculation with eggs is thymus-dependent.

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非経口的虫卵投与マウスにみられる小形条虫感染阻止能

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小形条虫虫卵が経口的に投与されるとマウス腸絨毛内で擬のう尾虫が発育してくる．このように，擬のう尾虫の絨毛内に存在することが虫卵再投与による再感染の成立を強く阻止するのに重要な役割を果している．しかし非経口的に虫卵を与えたとき，その後の虫卵の経口投与による感染が阻止されるかどうかはまだ調べられたことがない．マウス皮下に無菌的に注射された脱殻卵はその注入部位で擬のう尾虫にまでは発育できる．このような非経口的虫卵投与マウスを用意し，このマウスの虫卵経口感染阻止能を調べた．

生後1週目の ICR 系幼若マウス皮下へ脱殻卵を注射した場合，虫卵数5,000注射群では，注射後1週目に虫卵の経口投与を受けたすべてのマウスで経口感染は阻止されたが，注射後3週目に虫卵の経口投与を受けたマウスでは，すべてのもので経口感染が成立した．虫卵数50,000注射群では，1週齢目に注射その3週後に虫卵を経口投与されたマウスの大部分（15匹中13匹）でその感染は阻止されたが，同じ虫卵数50,000個を4週齢目に皮下注射その 1～3 週後に経口投与したときには，大部分のマウスで経口感染を阻止することができなかつた．また凍結乾燥した死滅卵を皮下注射した場合も，経口感染を阻止することはできなかつた．さらに，新生時に胸腺を摘出し1週齢に皮下に虫卵を注射したマウスでは，注射虫卵数の多少にかかわらず感染阻止能はみられず，ほとんどのマウスで経口感染が成立した．母親マウスを含めて，用いたすべてのマウスの腸管内には皮下注射時の虫卵汚染を疑わせるような成虫の寄生は全く認めず，皮下注射された虫卵はその注入部位で擬のう尾虫となつて検出された．

擬のう尾虫が皮下注射部位にとどまつたままになつているマウスの腸管における感染阻止能を調べて得た上記の結果は，その阻止能が生後早期に虫卵注射を行つた方がより効果的に誘導されてくることを示した。このことは腸管免疫の局所性がマウスの発育に伴つて発達完成してくることを暗示するものかも知れない．