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CHARACTERIZATION OF KINETICS OF ANTI-*TRICHINELLA SPIRALIS* NEWBORN LARVAE IMMUNITY IN RATS

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1. ABSTRACT

The kinetics of anti-*T. spiralis* newborn larvae (NBL) immunity and its dose effects were studied *in vivo*. Rats were either immunized with newborn larvae i.v. or muscle larvae *per os* and challenged with newborn larvae either i.v. or i.p. on day 7 up to day 27 after immunization. Immunity was assessed by examining the muscle larvae burden or the larval recovery from the peritoneal cavity. Recovered newborn larvae were further examined for cell adherence and viability. Results indicate that as early as 9 days after infection and only 3 days after newborn larvae production *in vivo*, specific anti-newborn larvae immunity was developed. Peritoneal cells as well as blood cells adhered to the cuticles of the larvae and killed them. When different doses of immunization were examined, it was found that 2,000 muscle larvae *per os* induced the strongest immunity as compared to 500, 5,000 or 6,000. Such immunity maintained its strength when challenge infection with newborn larvae reached 50,000 dosage and it declined significantly when the dose reached 100,000. This indicates that the immune cells and antibodies are not re-deployed.

2. INTRODUCTION

Thichinella spiralis is a highly immunogenic nematode parasite that can elicit strong and protective immune responses directed at various stages of the parasite in its mammalian host (1-8). Although most of the studies have been focused on host immunity against the adult and muscle larvae stages, it has been established that anti-newborn larvae immunity also plays an important role in the overall immune protection generated by the host and can be extremely effective in preventing newborn larvae from establishing in striated muscles (9-18). The kinetics of anti-newborn larvae immunity has been mainly studied by examining the time-course of cuticular cell adherence after *in vitro* incubation of newborn larvae with host leukocytes and immune serum obtained from rats (19,20) or mice (21) after a primary *T. spiralis* infection. Positive immune serum-mediated cell-attachment to newborn larvae was first detectable on day 15 or 30 in rats or mice, respectively. However, Bell *et al*. (22) demonstrated that in DBA/1 and AKR mice, sufficient immunity was generated after an i.v .injection of newborn larvae to provide 96-98% protection against a newborn larvae challenge given on day 10. These results indicate that in mice, anti-newborn larvae immunity can occur quite quickly after exposure to newborn larvae antigen. Whether rats have similar immune response patterns *in vivo* needs to be studied. There has been considerable evidence of larval killing via the antibody-dependent cell mediated cytotoxicity(23-26). Most except a

few (17,18) were carried out *in vitro*. Once more, the kinetics of such response was not studied.

As yet, no information is available on the dose-response characteristics of anti-newborn larvae immunity. However, the effects of different numbers of *T. spiralis* intestinal worms on anti-adult and anti-fecundity immune responses have been studied (6,27-33). The results of these studies show that worm dose is an important factor that influences the expression of host immunity. Both newborn larvae (34) and muscle larvae (35) have been shown to be immunosuppressive and it is possible that suppression could also be affected by larvae dose.

In this study, the following aspects of anti-newborn larvae immunity were examined: 1). The kinetics of rat anti-newborn larvae immunity *in vivo*; 2). The effect of different doses of newborn larvae or muscle larvae on anti-newborn larvae immunity; 3). The effect of various doses of newborn larvae in a challenge infection on expression of immunity.

3. MATERIALS AND METHODS

3.1. Experimental animals and infection

Adult male or female inbred AO rats weighing 150 to 250 g were used in these experiments and they were bred pathogen-free at the J. A. Baker Institute vivarium. Within one experiment, rats of only one sex were used. *T. spiralis* was maintained by serial passage in irradiated rats as donors. For infections with muscle larvae, from 500 up to 6,000 infective larvae, isolated by pepsin-hydrochloric acid digestion of infected muscles obtained from the donor rats, were administered orally to each rat. For infections with newborn larvae, 20,000 of the newborn larvae were freshly collected by incubating 6-day-old adult worms obtained from the small intestines of the donor rats for 24 hr in culture medium at 37^{o}C, separated from the adult worms after incubation, and then injected i.v. to each rat. For challenge infections, newborn larvae of various doses ranging from 10,000 to 100,000 were injected either i.p. or i.v. into each rat.

3.2. Examination of muscle larvae burden

The muscles were ground and digested overnight in 1,500 ml of 1 % pepsin in 1% HCl acid at 37^{0}C. The digestant was poured through cheesecloth and passed through a No. 200 mesh sieve to retain the larvae. After rinsing larvae from the screen of the sieve, they were collected and counted under a dissecting microscope.

3.3. Recovery of newborn larvae from peritoneal fluid and portal vein blood

To recover NBL from the peritoneal cavity of the rats, infected or challenged rats were injected i.p. with 10 ml of heparinized 0.85% saline warmed to 37^{0}C. The abdomen of the rat was massaged briefly, opened, and the fluid aspirated. The peritoneal fluid was centrifuged at 300 x g, and NBL were resuspended in 5 ml of 0.85% saline for enumeration. To recover NBL from portal vein blood, 1 ml of portal vein blood was taken and added to a test tube containing 1 ml of heparinied medium. After laking the blood with distilled water, the pellet was resuspended in 1 ml of 0.85% saline for counting. Since the number of larvae in 1 ml of blood sample rarely reached 50, the whole sample was examined. The possibility that some larvae might be lost by hypotonic shock in distilled water was investigated by placing NBL in distilled water for as long as 5 hr. No swelling or bursting was observed.

3.4. Determination of cell adherence to newborn larvae and viability of newborn larvae

In normal rats, cell adherence did not occur on more than 96% of larvae recovered from the peritoneal cavity 6 hr after injection. Less than 0.5% of the larvae recovered from the peritoneal cavity had 3 cells on their cuticle and no larva had more than 3 adherent cells. Based on this result, a finding of 4 or more cells adhering to NBL was considered positive adherence. Anything less was considered non-specific binding. To determine the viability of newborn larvae bearing host cells, larval motility was observed. Since live larvae move actively and constantly and dead larvae are straight and non motile, larvae lacking motility for 10 seconds and having a straightened appearance were recorded as dead.

3.5. Quantitation of immune protection against newborn larvae challenge

In the experiments performed here, when no significant difference ($p>0.05$) was found between muscle larvae burdens of experimental group rats and immunization control rats, this was taken to indicate that the experimental rats were 100% protected from the challenge infection. When the probability value was less than 0.05 between experimental and control groups, the percent immune protection against newborn larvae challenge was calculated as follows:

$$\text{Immune protection against newborn larvae challenge}(\%) = 1 - \frac{\text{Muscle larvae burden of group A rats} - \text{Muscle larvae burden of group 5 rats}}{\text{Muscle larvae burden of group B rats}} \times 100$$

3.6. Kinetics of cuticular cell adherence estimated *in vitro* and *in vivo*

AO rats were randomized into 11 groups of 3-4 rats. On day 1, 8 groups of rats were infected with 2,000 muscle larvae. Then, 7, 9, 10, 12, 13, 16, 18 and 21 days after infection, blood samples of 1 ml were taken from the tail vein of rats in one group per day. Immediately after blood collection, 20,000 newborn larvae were injected into the peritoneal cavity of each rat and then 6 hr later, peritoneal fluid was obtained and examined for newborn larvae. To determine cuticular adherence of blood leukocytes, individual blood samples were added to test tubes containing 0.5 ml of heparinized 0.85% saline and, after addition of 500 newborn larvae, the tubes were incubated at 37^{0}C on a hematology mixer (Fisher Scientific Co., U.S.A.) for 6 hr. Each blood sample was then laked with distilled water and examined microscopically for newborn larvae. Rats in 3 control groups were examined on days 7, 14 and 21 with identical experimental procedures.

3.7. Effect of immunization dose on anti-newborn larvae immunity

Rats (16) were randomized into four groups, 1 control and 3 immunized. The three immunized groups were

Table 1. The Effects of Immunization with Newborn Larvae on Immune Protection

GROUP	TREATMENT		ML BURDEN	% PROTECTION
	Immunization	**Challenge**		
5	20k(d1)	0	13,330±2,911	
A1	20k(d1)	20k(d8)	36,400±13,297	0
B1	0	20k(d8)	18,360±1,939	
A2	20k(d1)	20k(d15)	25,240±5,916	30
B2	0	20k(d15)	16,920±2,314	
A3	20k(d1)	20k(d22)	18,545±4,231	68
B3	0	20k(d22)	16,525±1,045	
A4	20k(d1)	20k(d27)	13,109±4,381	100
B4	0	20k(d27)	16,305±1,520	

On day 1, rats in all A groups and group 5 were injected i.v. with 20,000 NBL. Rats in groups 1-4, including both A and B groups were challenged i.v. with 20,000 NBL on day 8, 15, 22, 27, respectively. The muscle larvae burden of these rats was examined 20 days after challenge infection (for group 5, it was examined 20 days after immunization infection). The percent protection against the challenge infection with NBL was calculated according to the formula in Materials and Methods. No difference ($p>0.05$) was found between group 5 and group A4 rats. Muscle larvae burdens of all other group A rats were significantly different ($p<0.05$) from group 5 rats. Data represent means ± 1 standard deviation for 5 AO rats.

infected respectively with 500, 2,000 and 5,000 *T. spiralis* muscle larvae on day 1. On day 9, 5 ml of sterile 0.85% saline containing heparin (20 u/ml) was injected into the peritoneal cavity of individual immunized and control rats that had been lightly anesthetized with ether. After a brief abdominal massage the peritoneal fluid was aspirated with a 5 cc syringe under sterile conditions. Each peritoneal fluid sample was centrifuged at 500 xg for 10 min at 4^0C and the supernatant was kept at room temperature for later usage. The cell pellet was resuspended in 1 ml of the saved peritoneal fluid supernatant and counted in triplicate using a Coulter Counter. The reason for doing this was to include other possible factors, e.g., antibodies (in the fluid or on the cells) in the *in vitro* incubation experiment so that the results of this experiment would be directly comparable to the *in vivo* examination done on day 16. When the cell concentration in all 16 test tubes was determined, the one that had the lowest cell concentration, 3.4×10^6 cells/ml (obtained from a rat in the immunized group infected with 5,000 muscle larvae) was selected as the standard. The original peritoneal fluid was used to adjust cell concentration in all other test tubes to 3.4×10^6 cells/ml. When the cell concentration of all 16 samples had been standardized, 2,000 newborn larvae in 0.1 ml of RPMI-1640 were added to each test tube and all were further incubated at 37^0C in 5% $C0_2$ in air. After 1, 2, 3 and 5 hr, 50 ul samples were taken from individual test tubes and examined microscopically for newborn larvae, cell adherence and larval killing.

To examine the effect of variation in immunization dose at a later stage of *T. spiralis* infection, the same rats used above were injected with 25,000 newborn larvae i.p. on day 16. After 6 hr, 10 ml of heparinized 0.85% saline was injected i.p. and peritoneal fluid of these rats was obtained after a midline incision on rats anesthetized with ether. Peritoneal fluid samples were examined for the same parameters described above.

3.8. Statistical analysis

Within experiments, individual groups contained from 4 to 10 rats. Tabular data were recorded as the arithmetic mean $\pm$ 1 standard deviation, unless otherwise stated. Data were analyzed with analysis of variance and paired means were compared using Student-Newman Keuls t test. Probability values equal to or less than 0.05 were considered significant.

4. RESULTS

4.1. Kinetics of host anti-newborn larvae immunity determined by muscle larvae burden

The following experiment was performed to determine the dynamics of the host immune response against newborn larvae challenge. Forty- five rats were randomized into 8 paired groups and 1 control, i.e., A1, B1, ... A4, B4, and 5 (see table 1). Rats in all A groups and group 5 were immunized by i.v. injection with 20,000 newborn larvae on day 1. On days 8, 15, 22 and 27, rats in groups 1, 2, 3 and 4 (including both A, the immune group, and B, the control for challenge infection) were challenged iv with 20,000 newborn larvae respectively. To quantitate immunity, muscle larvae burdens of all these rats were examined 20 days after challenge infection or after the only infection as in group 5.

As shown in table 1, no immunity was evident when a challenge infection was given 7 days after immunization (see groups A1, B1 and group 5), that is, the muscle larvae burden of group A1 rats (36,400 $\pm$ 13,297) was not statistically different from the combined muscle larvae burdens of group B1 rats, the challenge control (18,360 $\pm$ 1,939) and group 5 rats, the immunization control (13,330 $\pm$ 2,911). Significant protection was evident when challenges were given on days 15, 22 and 27. Immunity was 30, 68 and 100% effective against the challenge infection with newborn larvae at these time points, respectively.

4.2. Kinetics of newborn larvae recovery from the peritoneal cavity of immune rats

It was documented that when newborn larvae were injected into the peritoneal cavity of immunized rats, few newborn larvae were recovered (14). This was shown to be a specific anti-newborn larvae immune response (14,15,18). To study the kinetics of this immune response, 44 AO rats were randomized into 11 groups of 4 rats, i.e., 8 immunized and 3 control groups. The individual immunized groups were infected with 2,000 muscle larvae on day 1

Table 2. Kinetics of anti-newborn larvae immunity detected in the peritoneal cavity

GROUP	TREATMENT		% LARVAE RECOVERY	% NBL with cells	% NBL dead
	ML per os	NBL ip			
C1	0	20k (d7)	58.0± 1.0	3.5±2.4	1.9±1.4
I1	2k (d1)	20k (d7)	64.4± +8.4	10.5± 2.8	6.5±1.6
I2	2k (d1)	20k (d9)	48.2± 2.1	12.0± 2.5	9.2±3.1
I3	2k (d1)	20k (d10)	43.5± 1.9	33.1± 12.2	31.5± 12.5
I4	2k (d1)	20k (d12)	33.2± 1.1	31.3± 3.4	30.0± 3.6
C2	0	20k (d14)	64.0± 8.3	0.3±0.5	0.3±0.5
I5	2k (d1)	20k (d14)	30.0± 4.2	45.2± 4.2	44.2± 3.9
I6	2k (d1)	20k (d16)	26.3± 2.0	66.8± 7.8	66.0± 9.1
I7	2k (d1)	20k (d18)	28.4± 2.5	63.8± 2.4	62.7± 2.5
C3	0	20k (d21)	65.3± 9.9	0.3±0.5	0.3±0.5
I8	2k (d1)	20k (d21)	23.1± 1.6	65.4± 2.6	65.4± 2.6

Rats in eight immune groups were infected with 2,000 muscle larvae on day 1. Individual immune groups were challenged with 20,000 newborn larvae on days 7, 9, 10, 12, 14, 16, 18 and 21. Three control groups were also injected ip with the same dose of newborn larvae. Peritoneal fluid of these rats was examined 6 hr after injection. Data represent means ± 1 standard deviation of 4 rats per point. The probability value is >0.05 on day 7, but <0.0005 on days 14 and 21 for newborn larvae recovery. Significant difference in cell adherence and larval killing between control and immune rats was evident on days 7 ($p<0.005$), 14 ($p<0.0005$) and 21 ($p<0.0005$).

Table 3. Kinetics of anti-newborn larvae immunity detected in the blood

GROUP	TREATMENT		% NBL WITH CELLS	% NBL DEAD
	ML per os	NBL ip		
C1	0	20k (d7)	0±0	0±0
I1	2k (d1)	20k (d7)	3.2±1.6	2.9±1.5
I2	2k (d1)	20k (d9)	32.6± 7.1	31.7± 4.7
I3	2k (d1)	20k (d10)	26.8± 5.2	24.8± 5.8
I4	2k (d1)	20k (d12)	30.0± 7.5	28.6± 7.2
C2	0	20k (d14)	2.6±2.3	2.6±2.3
I5	2k (d1)	20k (d14)	27.4± 5.3	27.2± 5.0
I6	2k (d1)	20k (d16)	23.7± 3.1	23.2± 2.8
I7	2k (d1)	20k (d18)	10.9± 1.8	10.9± 1.8
C3	0	20k (d21)	2.0±2.0	2.0±2.0
I8	2k (d1)	20k (d21)	8.7±0.3	8.7±0.3

The treatment is described in table 2. From each of the rats used in this experiment, 1 ml of blood was obtained and it was incubated with 500 newborn larvae for 6 hr at 37^0C and then laked with distilled water for microscopic examination. Significant differences were found in cell adherence on days 7, 14 and 21, in larval killing on days 14 and 21. Data represent means ± 1 standard deviation for 4 rats per point.

and were challenged separately with 20,000 newborn larvae i.p. on days 7, 9, 10, 12, 14, 16, 18, and 21. On days 7, 14 and 21, one of the control groups was also challenged i.p. with the same dose of newborn larvae. The peritoneal fluid of these rats was obtained 6 hr after injection and was examined for newborn larvae microscopically. The data are summarized in table 2 and show that on days 7, 14 and 21, larvae recovery in control rats was from 58.0 $\pm$ 1.0% to 65.3 $\pm$ 9.9%. In immunized rats, however, the recovery rate started at 64.4 $\pm$ 8.4% on day 7 and declined thereafter. The recovery rate plateaued at the level of ~25% from day 16 to day 21. By day 21, It was 23.1 ± 1.6% which was only 35% of the recovery rate in control rats. Larvae recoveries from immunized and control rats were significantly different on day 14 ($p<0.0005$) and day 21 ($p<0.0005$) but not day 7 ($p>0.05$).

4.3. Development of cell adherence to newborn larvae in the peritoneal cavity of immune rats

While enumerating newborn larvae in the immunized and control rats in the above experiment, the percentage of newborn larvae recovered that had cells attached and the percentage of newborn larvae that were immobile were also determined. As shown in table 2, the percent of newborn larvae bearing cells was extremely low on days 7, 14 and 21 (3.5 ± 2.4, 0.3 ± 0.5, 0.3 $\pm$ 0.5, respectively) in control rats as was the number of dead larvae (1.9 $\pm$ 1.4, 0.3 ± 0.5 and 0.3 ± 0.5). In contrast, 10.5 ± 2.8% of newborn larvae recovered from immunized rats had adherent cells on day 7 ($p<0.005$). The number of dead larvae was 6.5 ± 1.6% ($p<0.005$). These parameters increased to 66.8 ± 7.8% (newborn larvae that had cell adherence) and 66.0 ± 9.1% (newborn larvae that were dead) on day 16, and continued at the same level until day 21, which was the last day examined. A significant difference in both parameters between control and immunized rats was evident on day 14 ($p<0.0005$) and day 21 ($p<0.0005$). Since from 62-100% of recovered larvae with cells attached were also dead, the kinetics of larvicidal activity correlated closely with cell adherence to newborn larvae.

4.4. Development of cuticular cell adherence after *in vitro* incubation with blood from immunized rats

To determine whether cuticular cell adherence and larvicidal activity occurred in the blood and its kinetics, blood samples of 1 ml were taken from the rats in the above experiment. Each sample was incubated with 500 newborn larvae in test tubes for 6 hr at 37^0C on an automatic mixer, the blood sample was then laked and examined microscopically. After incubation in the blood from control rats, few newborn larvae (0-2.6%) had cells attached or were dead (table 3). When newborn larvae were incubated in the blood taken from immunized rats, however, the percentage of newborn larvae with adherent cells increased dramatically as did the number of dead larvae. On day 9,

Table 4. Effects of different doses of muscle larvae on cell adherence to newborn larvae and larval killing

GROUP	DOSE OF mL INFECTION IN CELL DONORS	HOURS AFTER INCUBATION *IN VITRO*							
		1		2		3		5	
		With cells	Dead	With cells	Dead	With Cells	Dead	With cells	Dead
1	0	0±0	0±0	0±0	0±0	0.6±1.1	0±0	0±0	0±0
2	500	3.3±1.9	1.2±1.5	9.2±7.1	2.1±1.6	10.8±4.5	1.9±1.8	10.7±6.2	7.4±2.3
3	2,000	6.0±2.1	4.1±2.9	8.6±1.6	3.0±1.5	10.8±4.1	2.4±0.9	17.1±3.5	10.1±2.5
4	5,000	12.3±3.0	6.2±0.6	16.4±4.9	2.5±2.0	13.5±9.8	2.9±1.6	15.4±2.1	12.9±1.1
Probability	(1:2)	**	NS	**	**	**	*	**	**
	(1:3)	**	**	**	**	**	**	**	**
	(1:4)	**	**	**	*	**	**	**	**
	(2:3)	NS	NS	NS	NS	NS	NS	NS	NS
	(2:4)	**	**	NS	NS	NS	NS	NS	**
	(3:4)	**	NS	**	NS	NS	NS	NS	*

Immune cell donor rats were infected on day 1. The peritoneal cells of immune and control rats were obtained on day 9. These non-washed cells at a concentration of $3.4x10^6$ were incubated with 2,000 newborn larvae in the peritoneal fluid of cell donor rats. Samples were examined microscopically 1, 2, 3 and 5 hr after incubation. Data represent means ± 1 standard deviation of 4 rats per group. NS: not significant; *: $p<0.05$; **: $p<0.01$.

for example, an average of 32.6 ± 7.1% of the larvae had peritoneal cells on the cuticle and 31.7 ± 4.7% were dead. Similar results were obtained on days 10, 12 and 14.

Beginning on day 16, some larvae that had cells attached to them after incubation disintegrated after laking the blood with distilled water. Partial remains of larvae with adherent cells were often observed. These were not countable and, therefore, led to a reduction in the numbers of cell-coated larvae and dead larvae examined after day 16. By day 21, only 8.7 ± 0.3% of the recovered larvae that had adherent cells were still intact and all of these larvae were dead. Differences between control and immunized groups in cell adherence to newborn larvae were significant on days 7,14 and 21 and in larval killing on days 14 and 21.

4.5. The influence of dose of muscle larvae on anti-newborn larvae immunity

This experiment was designed to determine whether a high dose of muscle larvae affected the expression of anti-newborn larvae immunity. The experiment comprised 3 groups of 3 rats, those in groups 2 and 3 were infected on day 1 with 2,000 and 6,000 muscle larvae, respectively. Group 1 rats were not infected and were controls for groups 2 and 3. Fourteen days after infection, all rats were challenged i.p. with 14,400 newborn larvae and the peritoneal fluid of these rats was examined for newborn larvae 6 hr after injection. The results show (figure 1a) that the highest recovery (57.4 ± 3.5%) was obtained from control rats, the lowest (19.2 ± 3.8%) from the rats infected with 2,000 muscle larvae; from the rats infected with 6,000 muscle larvae, the recovery was in between (34.7 ± 13%). No statistical difference was established in the results obtained from two immunized groups but both of these results were significantly different from the controls ($p<0.0005$, $p<0.025$). In contrast to the above findings, the lowest percentages of newborn larvae bearing cells (0.8 ± 0.7%) and newborn larvae that were dead (0.4 ± 0.8%) were found in the control group (figure 1b,c) and the highest percentages of both parameters (99.0 ± 1.7%) were obtained from rats infected with 2,000 muscle larvae. Again, rats infected with 6,000 muscle larvae gave the intermediate results (49.2 ± 32.2%). Both immunized groups were significantly different from controls and also from each other.

4.6. The effects of different doses of muscle larvae infection on anti-newborn larvae immunity detected on day 9 and day 16 of *T. spiralis* infection

To determine whether the reduction of anti-newborn larvae immunity caused by high dose muscle larvae infection also took place at an earlier stage of newborn larvae production *in vivo*, the following experiment was carried out. Sixteen rats were randomized into 4 groups, i.e., 1 control and 3 immunized. Three immunized groups were infected with 500, 2,000 or 5,000 muscle larvae on day 1. Eight days later, the peritoneal fluid of immunized rats and the controls was obtained under sterile conditions. The non-washed peritoneal cells in the fluid (3.4×10^6) from individual rats were incubated with 2,000 newborn larvae and the samples were examined microscopically 1, 2, 3 and 5 hr after incubation. On day 16, the same rats were challenged ip with 25,000 newborn larvae and the peritoneal fluid was examined 6 hr after injection.

It was found (table 4) that compared with the controls (0-0.6%), peritoneal cells from all 3 immunized groups rendered significantly higher percentages of cell-bearing larvae (3.3 to 17.1 %) and larval mortality (1.2 to 12.9%) at any of the time intervals examined. Generally, the differences between 3 immunized groups were not statistically significant. The results of the later stage examination (figure 2) show that the lowest percentage of larvae recovery (20.3 ± 12.8%) and highest percentage of larvae carrying cells (86.7 ± 20.6%) and larval killing (45.5 ± 22.5%) were found in rats infected with 2,000 muscle larvae 15 days previously. In rats infected with 500 or 5,000 muscle larvae, however, larvae recovery increased to 59.4 ± 13% and 47.0 ± 24.6% respectively. The number of newborn larvae with adherent cells or dead decreased to 25.8 ± 19.0% and 15.9 ± 11.4% in rats infected with 500

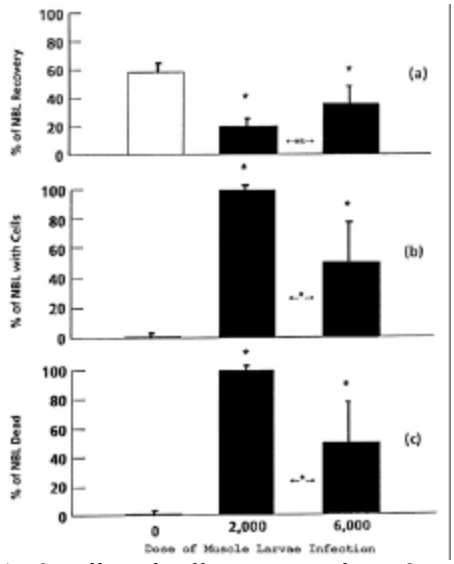

Figure 1. The Effect of Different Doses of Muscle Larvae Infection on Anti-Newborn Larvae Immunity. On day 1, rats in two immune groups (solid bars) were infected with 2,000 or 6,000 muscle larvae, respectively. On day 15, immune and control (open bars) rats were injected i.p. with 14,400 newborn larvae and their peritoneal fluids were examined 6 hr later. Data represent means ± 1 standard deviation for 3 rats per group. *: $p<0.05$ when comparing individual immune groups with the control or comparing two immune groups. NS: not significant.

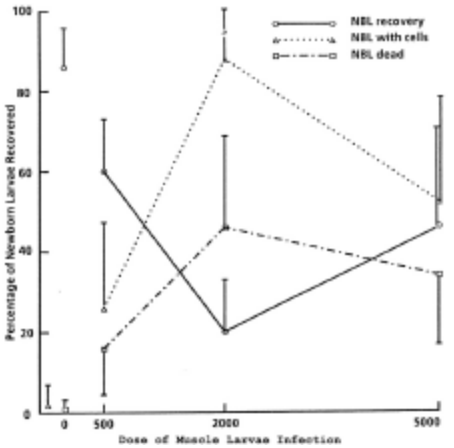

Figure 2 The Effects of Different Doses of Muscle Larvae Infection on Anti-Newborn Larvae Immunity Examined on Day 16 of *T. spiralis* Infection. Rats were infected with different doses of muscle larvae on day 1 (0, 500, 2,000 and 5,000, respectively) and were challenged i.p. with 25,000 newborn larvae on day 16. Peritoneal fluid of these rats was examined 6 hr after injection. Results in all 3 immune groups were significantly different ($p<0.05$) from the controls. No difference ($p>0.05$) was found in all parameters examined between rats infected with 500 or 5,000 muscle larvae. Significant difference ($p<0.05$) was established in all parameters from rats infected with 500 or 2,000 muscle larvae, and in one parameter (newborn larvae with cells) between groups that were infected with 2,000 or 5,000 muscle larvae.

muscle larvae and to 51.9 ± 26.1, 33.8 ± 15.4% in rats infected with 5,000 muscle larvae

4.7. The effect of different doses of newborn larvae challenge on anti-newborn larvae immunity detected in the peritoneal cavity

To determine the dose effect of challenge infection, rats were divided into 3 control and 3 immune groups, 3 to 4 rats per group. The immunized groups were infected with 2,000 muscle larvae on day 1. The challenge infection of newborn larvae was given i.p. with different doses (10,000, 50,000 and 100,000 newborn larvae per rat) to individual immunized groups and their controls on day 16. The peritoneal fluid was examined 6 hr after i.p. injection. From the peritoneal fluid of 3 immunized groups, larvae recoveries were significantly decreased compared with their controls (table 5). The average number of larvae recovered from the immunized rats was less than 200 (<2.0% of the injected dose) even from rats challenged with 100,000 newborn larvae. In contrast, larvae recovery in control rats ranged from 36 to 85% of the injected dose (table 5) and neither cell attachment nor larval killing was observed (figure 3). In immunized rats challenged with 10,000, 50,000 and 100,000 newborn larvae, however, 88.9 ± 19.2, 86.1 ± 16.7 and 34.8 ± 13.8 percent respectively, of the recovered larvae had adherent cells on their cuticle and 95.2 ± 8.3, 83.4 ± 19.2 and 29.5 ± 15% of the recovered larvae were dead, respectively (figures 3, 4a,b). All these results were significantly different from their controls ($p<0.05$). Cell adherence to newborn larvae and larval mortality for immunized rats challenged with 100,000 newborn larvae were also significantly lower ($p<0.005$) than for immunized rats challenged with 10,000 or 50,000 newborn larvae (figure 3). For example, in immune rats challenged with 100,000 newborn larvae, the percentage of recovered larvae with cells attached (34.8 ± 13.8%) was only around 40% of that in immunized rats challenged with 10,000 (88.9 ± 19.2%) or 50,000 (86.1 ± 16.7%) newborn larvae. Larval mortality (29.5 ± 15.0%) in this group (challenged with 100,000 larvae) was only 31-35% of that in the other two immunized groups.

5. DISCUSSION

The experiments reported here systematically studied the kinetics of anti-newborn larvae immunity. Two approaches were employed in the experiments. First, the dynamics of the immune response were determined by comparing muscle larvae burdens of rats immunized with newborn larvae i.v. and then challenged with newborn larvae i.v. at various intervals after immunization. It was found that the onset of immune protection took place 1 week after immunization (table 1), which confirms the findings in mice by Bell *et al*. (22). The peak incidence of this immunity was at 4 weeks after newborn larvae immunization when 100% protection was established.

The second approach involved immunizing rats with muscle larvae and then detecting anti-newborn larvae immunity directly in the peritoneal cavity of rats after challenging them with newborn larvae i.p. In addition,

Table 5. The effect of different dose of newborn larvae challenge on anti-newborn larvae immunity

	TREATMENT		NEWBORN LARVAE	
Group	ML(per os) immunization	NBL(ip) challenge	Recovery	% of ip injection
Control	0	10k	3,547± 1,382	35.5± 13.8
Immunized	2k	10k	183± 113	1.9± 1.1
Control	0	50k	42,219±2,528	84.5± 5.1
Immunized	2k	50k	125± 84	0.3± 0.2
Control	0	100k	79,688± 14,210	79.7± 14.2
Immunized	2k	100k	194± 109	0.2± 0.1

The rats in immunized groups were infected on day 1. All rats were then challenged on day 22. The peritoneal fluid of these rats was examined 6 hr after i.p. injection of newborn larvae. Data represent means ± 1 standard deviation of 3-4 rats per group. The results of all parameters in immunized rats are significantly ($p<0.05$) different from those in control rats.

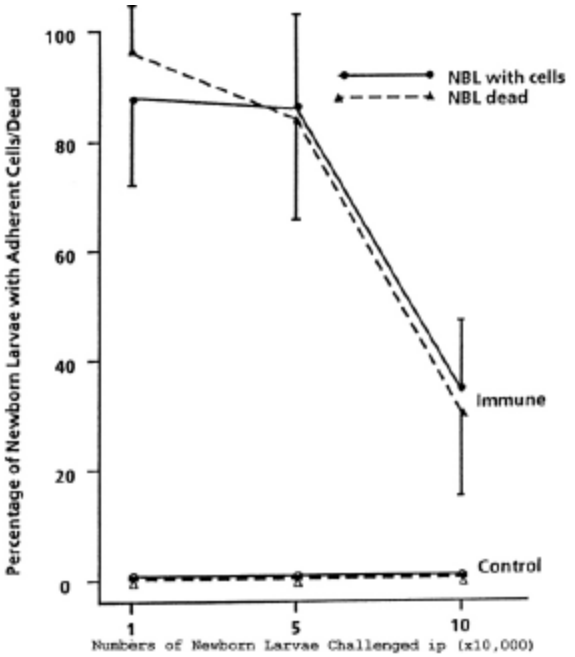

Figure 3. The Effect of Different Doses of Challenge Infection on Anti-Newborn Larvae Immunity. Rats in three immune groups were infected with 2,000 muscle larvae on day 1. These rats and the controls were challenged i.p. with 10,000, 50,000, and 100,000 newborn larvae respectively on day 16. Peritoneal fluid was examined 6 hr after injection. Data represent means ± 1 standard deviation for 3-4 rats per point. The results of all immune groups are significantly different ($p<0.05$) from their controls. The results of immune rats challenged with 100,000 newborn larvae are also significantly different ($p<0.0005$) from immune rats challenged with 10,000 or 50,000 newborn larvae.

blood samples of these rats were also incubated with newborn larvae *in vitro*. It was found that 8 days after muscle larvae infection, newborn larvae recovery from the peritoneal cavity was considerably reduced (table 2). The cell adherence and larvicidal activity observed after i.p. injection of newborn larvae or after incubation of larvae with blood, however, were greatly increased compared to the controls (tables 2 and 3). These effects peaked on day 16 (table 2) for peritoneal cells and as early as on day 9 (table 3) for blood cells. The onset of anti-newborn larvae immunity detected here is 7 to 14 days earlier than the *in vitro* findings by Mackenzie *et al.* (19) and Philipp *et al.* (20), which suggests that the *in vitro* assay may not be sensitive enough to reflect an immunity which is expressed *in vivo.*

The lag phase of immune response against newborn larvae is 6 days shorter in rats immunized with muscle larvae *per os* (tables 2 and 3) than in rats immunized with newborn larvae i.v. (table 1). Newborn larvae are first produced by adult *T. spiralis* worms at around day 6 post infection (36). When the anti-newborn larvae immune response is detectable on day 9 after muscle larvae infection, by this time, the host immune system has only been stimulated by newborn larvae for no more than 3 days. When rats are immunized i.v. with newborn larvae, however, they need at least 9-14 days to trigger the host immune system to respond (table 1). In addition, after muscle larvae immunization, the anti-newborn larvae response reaches maximum activity 11 to 18 days earlier (5 to 12 days after newborn larvae production, Tables 2 and 3) than in rats immunized with newborn larvae i.v. (26 days post inoculation, table 1).

It is known that newborn larvae are continuously produced by adult worms in the small intestine of rats from day 6 onwards until the adult worms are rejected (36). During this period, due to the systemic larval dissemination and recirculation (37), newborn larvae antigens are extensively exposed to the host immune system so that the immune response is expected to be stronger. In contrast, when immunization is given by a single i.v. injection with newborn larvae, the exposure of the larval antigens to the host is significantly shortened (14). Hence, one is expected to observe a longer lag phase before the onset of the immune response and it requires a more extended period to mount the maximum anti-newborn larvae activity. Another possibility is that after muscle larvae infection the small intestine of rats is sequentially stimulated by muscle larvae, adult worms and newborn larvae and this may enhance the immune response against newborn larvae.

When newborn larvae were incubated with the blood obtained from rats 14 days after muscle larvae infection, cell adherence to newborn larvae and the larvicidal effect seemed on the decline (table 3). The most likely reason is that by this time, newborn larvae that were bearing cells could no longer stand the laking treatment, i.e., they were lysed, and therefore, they were not counted.

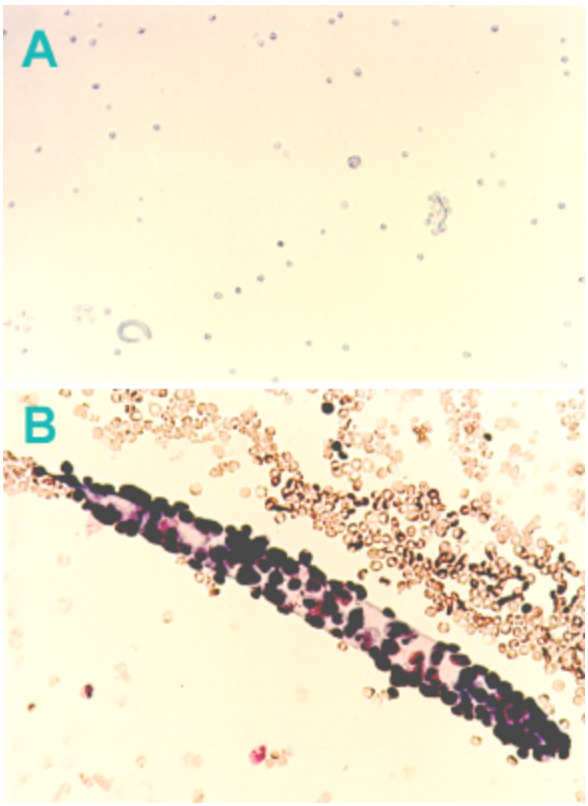

Figure 4. Newborn lavae with cell adherence. (a) One with cells attached, one without. (b) A closer view of the cell adherence.

This phenomenon actually indicates a stronger larvicidal response of the host.

The effects of different doses of immunization and challenge infection on the expression of anti-newborn larvae immunity were studied. The results demonstrate that a relatively weak anti-newborn larvae immunity is incident to the low immunization dose (500 muscle larvae) given (figure 1). When the immunization dose is high (5-6,000 muscle larvae *per os*), however, the effect of anti-newborn larvae immunity is not proportionally increased. On the contrary, it is decreased dramatically (figures 2, 3). The reduction of anti-newborn larvae immunity does not occur at an earlier stage (day 9) (table 4) but takes place at a later time (day 16) during the infection (figure 2). These results suggest that through low dose immunization, host immune system may not be sufficiently stimulated. High dose immunization, on the other hand, may induce a suppressive effect on host immunity. At an earlier stage of infection, e.g., 8 days after muscle larvae infection, newborn larvae that have been produced have not developed to muscle larvae. By day 16, however, many newborn larvae are maturing to muscle larvae in the striated muscle cells. Hence, it is likely that the maturing muscle larvae may be responsible for this suppressive effect. At the moment, it is not clear what the mechanism is for this suppressive effect. Compared to the low and high dose immunization, the most optimal immunization dose examined is 2,000 muscle larvae infection *per os* (figures 1, 2) or 20,000 newborn larvae injection i.v. (table 1), both of which induce the strongest immune response against newborn larvae.

The results of challenge dose response on anti-newborn larvae immunity demonstrate that the higher the challenge dose is given, the lower the effects of cell adherence to newborn larvae and larval killing are observed (figure 3). These data suggest that first, the effectors of the immunity may be diluted by the large numbers of newborn larvae given in the challenge infection, and second, the effectors may not be reused. This means that once the antibodies and effector cells attach to the target larvae, they do not detach from these targets and reattack other larvae. In fact, the experimental observations proved this hypothesis. When placing the cell-coated larvae obtained from the peritoneal fluid of immune rats on a slide and pressing the slide with a cover slip, the outer layers of cells could be pressed away from the larvae. Nonetheless, the inner-layer cells never became loosened from the larvae (figure 4).

Summarizing, the results described here demonstrate that anti-newborn larvae immune response is generated in rats 3-4 days after newborn larvae production during a primary *T. spiralis* infection. This response occurs within 2 weeks after i.v. injection of newborn larvae. Low dose (500 muscle larvae) immunization elicits a sufficient yet not strong anti-newborn larvae immunity. High dose (5-6,000 muscle larvae *per os*) immunization leads to a reduction of the immune effect against newborn larvae. The suppressive effect induced by high dose immunization with muscle larvae is not evident on day 9 of *T. spiralis* infection but on day 16. The most optimal immunization dose examined is 2,000 muscle larvae. Due to the fact that the immune effectors once attached to the first larva, do not re-deploy themselves to other unattended target larvae, high dose challenge infection reduces the effect of anti-newborn larvae immunity.

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7. REFERENCES

1. E. R. James & D. A. Denham: Immunity to *Trichinella spiralis*. VI. The specificity of the immune response stimulated by the intestinal stage. *J Helminthol* 49, 43-47 (1975)

2. E. R. James, A. Moloney & D. A. Denham: Immunity to *Trichinella spiralis*. VII. Resistance stimulated by the parenteral stages of the infection. *J Parasitol* 63, 720-723 (1977)

3. E. J. Ruitenberg & P. A. Steerenberg: Immunogenicity of the potential stages of *Trichinella spiralis*. *J parasitol* 62, 164-166 (1976)

4. D. D. Despommier: Biology. In: Trichinella and Trichinosis. Ed: Campbell W. C., Plenum Press, NY and London (1983)

5. R. J. Love, B. M. Ogilvie & D. J. McLaren: The immune mechanism which expels the intestinal stage of *Trichinella spiralis* from rats. *Immunol* 30, 7-15 (1976)

6. R. G. Bell, D. D. McGregor & D. D. Despommier: *Trichinella spiralis*: Mediation of the intestinal component of protective immunity in the rat by multiple, phase-specific, antiparasitic responses. *Exp Parasitol* 47, 140-157 (1979)

7. J. A. Appleton & D. D. McGregor: Life-phase specific induction and expression of rapid expulsion in rats suckling *Trichinella spiralis*-infected dams. *Immunol* 55, 225-232 (1985)

8. J. E. Larsh Jr., A. Ottolenghi & N. F. Weatherly: *Trichinella spiralis*: Phospholipase in challenged mice and rats. *Exp Parasitol* 36, 299-306 (1974)

9. T. D. G. Lee & D. Befus: Effects of rat and human intestinal lamina propria cells on viability and muscle establishment of *Trichinella spiralis* newborn larvae. *J Parasitol* 75, 124-128 (1989)

10. D. D. Despommier: Immunogenicity of the newborn larva of *Trichinella spiralis*. *J Parasitol* 57, 531-535 (1971)

11. D. A. Denham & A. R. Martinez: Studies with methyridine and *Trichinella spiralis*. 2. The use of the drug to study the rate of larval production in mice. *J Helminthol* 44, 357-363 (1970)

12. A. Moloney & D. A. Denham: Effects of immune serum and cells on newborn larvae of *Trichinella spiralis*. *Parasite Immunol* 1, 3-12 (1979)

13. K. D. Murrell: *Trichinella spiralis*: Acquired immunity in swine. *Exp Parasitol* 59, 347-354 (1985)

14. C. H. Wang & R. G. Bell: *Trichinella spiralis*: Host immunity against newborn larvae during a primary infection in rats. *J Parasitol* 73, 1069-1071 (1987)

15. C. H. Wang & R. G. Bell: *Trichinella spiralis*: Intestinal expression of systemic stage-specific immunity to newborn larvae. *Parasite Immunol* 9, 465-475 (1987)

16. C. H. Wang & R. G. Bell: Host immunity against newborn *Trichinella spiralis* larvae of different ages. *J Parasitol* 74, 880-882 (1988)

17. C. H. Wang & R. G. Bell: Antibody-mediated *in vivo* cytotoxicity to *Trichinella spiralis* newborn larvae in immune rats. *Parasite Immunol* 10, 293-308 (1988)

18. C. H. Wang & R. G. Bell: Characterization of cellular and molecular immune effectors against *Trichinella spiralis* newborn larvae *in vivo*. *Cell Molecular Biol* 38, 311-325 (1992)

19. C. D. Mackenzie, P. M. Preston & B. M. Ogilvie: Immunological properties of the surface of parasitic nematodes. *Nature* 276, 826-828 (1978)

20. M. Philipp, P. M. Taylor, R. M. E. Parkhouse & B. M. Ogilvie: Immune response to stage-specific surface antigens of the parasitic nematode *Trichinella spiralis*. *J Exp Med* 154, 210-215 (1981)

21. M. Jungery & B. M. Ogilvie: Antibody response to stage-specific *Trichinella spiralis* surface antigens in strong and weak responder mouse strains. *J Immunol* 1129, 839-843 (1982)

22. R. G. Bell, C. H. Wang & R. W. Ogden: *Trichinella spiralis*: Nonspecific resistance and immunity to newborn larvae in inbred mice. *Exp Parasitol* 60, 101-110 (1985)

23. J. W. Kazura & M. Aikawa: Host defense mechanisms against *Trichinella spiralis* infection in the mouse: eosinophil-mediated destruction of newborn larvae *in vitro*. *J Immunol* 124, 355-361 (1980)

24. C. D. Mackenzie, M. Jungery, P. M. Taylor & B. M Ogilvie: Activation of complement, the induction of antibodies to the surface of nematodes and the effect of these factors and cells on worm survival *in vitro. Europ J Immunol* 10, 594-601 (1980)

25. S. M. Venturiello, G. H. Giambartolomei & S. N. Costantino: Immune cytotoxic activity of human eosinophils *against Trichinella spiralis* newborn larvae. *Parasite Immunol* 17, 555-559 (1995)

26. S. M. Venturiello, S. N. Costantino & G. H. Giambartolomei: Blocking anti-*Trichinella spiralis* antibodies in chronically infected rats. *Parasite Res* 82, 77-81 (1996)

27. O. R. McCoy: Size of infection as an influence on the persistence of adult Trichinae in rats. *Science* 75, 364-365 (1932)

28. O. F. Gursch: Intestinal phase of *Trichinella spiralis* (Owen, 1835), Railliet 1895. *J Parasitol* 35, 19-26 (1949)

29. C. Khamboonruang: Output of larvae and lifespan *of Trichinella spiralis* in relation to worm burden and superinfection in the mouse. *J Parasitol* 57, 289-297 (1971)

30. C. E. Tanner: The susceptibility to *Trichinella spiralis* of inbred lines of mice differing at the H-2 histocompatibility locus. *J Parasitol* 64, 956-957 (1978)

31. R. G. Bell & D. D. McGregor: *Trichinella spiralis*: Role of different life cycle phases in induction, maintenance, and expression of rapid expulsion in rats. *Exp Parasitol* 48, 51-60 (1979)

32. R. G. Bell & D. D. McGregor: *Trichinella spiralis*: Expression of rapid expulsion in rats exposed to an abbreviated enteral infection. *Exp Parasitol* 48, 42-50 (1979)

33. R. G. Bell, D. D. McGregor, M. C. Woan & L. S. Adams: *Trichinella spiralis*: Selective intestinal immune deviation in the rat. *Exp Parasitol* 56, 129-142 (1983)

34. G. M. Faubert: Depression of the plaque-forming cells to sheep red blood cells by the new-born larvae of *Trichinella spiralis*. *Immunol* 30, 485-489 (1976)

35. T. Alkarmi, M. K. Ijaz, F. K. Dar, S. Abdou, S. Alharbi, P. Frossard & M. Narem: Suppression of transplant immunity in experimental trichinellosis. *Comp Immunol Microbiol Infect Dis* 18, 171-177 (1995)

36. C. H. Wang & R. G. Bell: *Trichinella spiralis*: Newborn larval migration route in rats reexamined. *Exp Parasitol* 61, 76-85 (1986)

37. C. H. Wang & R. G. Bell: *Trichinella spiralis*: Vascular recirculation and organ retention of newborn larvae in rats. *Exp Parasitol* 62, 430-441 (1986)

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