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Prolongation of Infection Time and Failure of Restoring Fecundity of Mouse-nonadaptive *Nippostrongylus brasiliensis* by Administrations of Cyclophosphamide or Anti-CD4 Antibody in Mice

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Abstract

Mouse-nonadaptive *Nippostrongylus brasiliensis*, which did not deposit eggs in adult mice, grew into intestinal worms in BALB/c mice. The intestinal worms were young adult and mainly recovered at 5 days post-infection (PI) and disappeared without egg production within 7 days PI. The mice treated with 150 or 300 mg/kg of cyclophosphamide one day before infection showed intestinal worm burden at 7 days PI, suggesting prolongation of infection time. The worms retained in the small intestine by a single injection of cyclophosphamide, however, were expelled within 13 days PI without depositing eggs. The worm expulsion was also prolonged by treatment with 2 mg of anti-CD4 antibody but not with anti-CD8 antibody. However, fecundity was not restored in the treated mice. These results suggest that mice infected with the nonadaptive *N. brasiliensis* are restored by the immune responses regulated by $CD4^+$ lymphocytes but fecundity of the worms may be regulated by additional factors.

Key words: *Nippostrongylus brasiliensis*, anti-CD4, cyclophosphamide, expulsion, fecundity, mice

Introduction

Nippostrongylus brasiliensis is naturally an intestinal parasite of rats and usually has low ability to develop in mice. However, the mouse-adaptive strain of *N. brasiliensis* has been established by serial passages in young mice of 4 to 5 weeks old for 5 to 7 generations (Wescott and Todd, 1966; Solomon and Haley, 1966) and maintained in adult mice (Solomon and Haley, 1966; Katona *et al.*, 1983). The adaptation to mice was induced by passing *N. brasiliensis* in immunologically immature young mice. Therefore, an escape from immune responses is probably important to give rise to the mouse-adapative strain. It might be derived from the rat-adaptive worms by changing their ability to develop in mice or by expanding a population of worms which already carried the ability to develop in mice.

We have maintained a strain of *N. brasiliensis*, which had been adapted to mice, by serial passages in rats for several years. The strain of *N. brasiliensis* has become nonadaptive to mice. That is, almost no egg is detected in the feces from infected adult BALB/c or C57BL/6 mice. This strain of *N. brasiliensis*, however, can develop to adult worms and deposit eggs in BALB/c (nu/nu) mice (Abe *et al.*, 1992). Adaptability of this strain to adult mice, therefore, seems to be regulated by the thymus dependent immune responses. Although T cell requirement for the spontaneous expulsion of *N. brasiliensis* is well established (Mitchell *et al.*, 1982; katona *et al.*, 1988), little is known about the regulation of adaptability to mice. We, therefore, examined if treatments with cyclophosphamide or antibodies to T cell subsets can modify the infection time or egg production of the *N. brasiliensis* in mice.

Materials and Methods

Animals. Original colonies of BALB/c mice, C57BL/6 mice and Wistar rats were purchased from Japan SLC Co. (Shizuoka, Japan). BALB/c and C57BL/6 mice were raised from the colonies in the

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Animal Center of Akita University School of Medicine and used at 8–17 weeks (BALB/c mice) or 4–5 weeks (C57BL/6 mice) old. All animals were treated properly in accordance with the Guideline for Animal Experimentation, Akita University.

Parasitological techniques. N. brasiliensis was provided by Dr. N. Watanabe (Tokyo Jikei University School of Medicine) and has been maintained in our laboratory for more than 5 years by serial passages in rats. Infective larvae were obtained by the fecal culture on charcoal, washed three times with saline and infected s.c. to mice by injecting 0.2 ml of a solution containing 400 larvae unless specially indicated. The methods for estimating egg number per gram feces (EPG) and recovery of intestinal worms were as reported previously (Abe *et al.*, 1992). Briefly, for worm recovery the whole small intestine was sectioned, opened longitudinally and incubated in saline containing 0.01% sodium hypochlorite at 37°C for 3 h. After incubation, the tissues in a tube were shaken and removed from the tube. The intestinal worms in the tube were washed twice and counted on a plate under a dissecting microscope. Worm length was measured using a Nikon profile projector, Model V-16C (Nippon Kogaku, Japan).

Histology. Pieces of the small intestine at about 10 cm from the pylorus were immersed in Carnoy's fixative or neutralized 10% formalin and sectioned as a paraffin block. The sections from Carnoy's fixative were stained with Alcian blue (pH 0.3) and Safranin-O (pH 1.0) for counting mucosal mast cells or with periodic acid-Schiff reaction for counting goblet cells. The sections from formalin fixative were stained with Biebrich scarlet red for counting eosinophils. Numbers of mucosal mast cells or eosinophils in the epithelium and villous lamina propria were counted together for 15–30 villus crypt units (VCU). Numbers of goblet cells were counted both in the villi and crypts.

Administration of cyclophosphamide. Mice were injected i.p. with 150 to 300 mg/kg of cyclophosphamide (Aldrich Co., WIS, USA) freshly prepared in 0.2–0.4 ml of sterile saline one day before infection.

Antibodies. Hybridomas producing rat IgG2b monoclonal anti-L3T4 (CD4) antibody, GK1.5, and mouse IgM monoclonal anti-Lyt2.2 (CD8) antibody, HO2.2, were obtained from the American Type Culture Collection (Rockvill, MD, USA). The immunoglobulin fractions were collected from the cell culture supernatants by precipitation with ammonium sulfate, dialyzed against phosphate buffered saline and stored at –30°C until used. Amount of immunoglobulin was corrected according to its percentage in the precipitate obtained by high performance liquid chromatography with a column of TSK-gel, G-3000SW (Toyo Soda, Japan). Mice were injected i.p. with 2 mg of anti-CD4 or anti-CD8 antibody one day before infection.

Statistical analysis. Probability of significant differences between groups ($P<0.05$) was determined by Mann-Whitney's U-test.

Results

Adult BALB/c or C57BL/6 mice of older than 7 weeks, which are infected with the strain of *N. brasiliensis*, deposited no egg in the feces. This was not due to inhibition of growth of intestinal worms, because intestinal worm burden was detected at 4 days PI, peaked at 5 days PI, then decreased and almost disappeared at 7 days PI in BALB/c (Fig. 1). A similar result was obtained in C57BL/6 mice (data not shown). Length of intestinal worms recovered from BALB/c mice was compared to that of worms recovered from rats at 5 days PI. Length of female worms from mice was 254±8 μm (mean ± SE, n=20) and that of male worms from mice was 224±5 μm (n=16). Length of female or male worms from rats was 390±4 μm (n=25) or 290±4 μm (n=22), respectively. Size of worms was significantly smaller in mice. They were young adult devoid of eggs in the uteri but not larvae. Sexual maturation of this strain of *N. brasiliensis* seemed to be delayed or inhibited in adult mice. In young C57BL/6 mice of 4 to 5 weeks old, however, this strain of *N. brasiliensis* deposited eggs in the feces (Table 1). Although long passages of the strain were unsuccessful in the mice, infective larvae could be obtained from those murine feces at 7 days PI.

To see if the short time infection can be modified by blocking host immune responses, BALB/c mice were treated with a single dose of cyclophosphamide one day before infection. Treatment of mice with 150 or 300 mg/kg of cyclophosphamide, which are

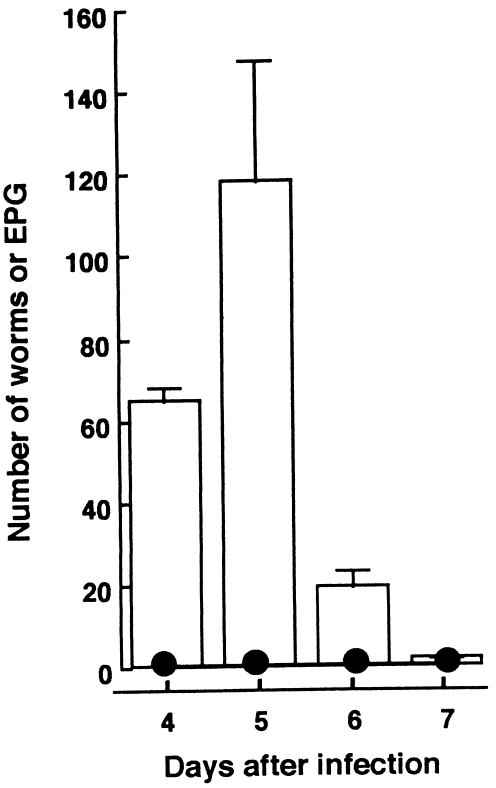

Fig. 1 Kinetics of intestinal worm burden and EPG in BALB/c mice. Twenty five female mice were infected with 400 larvae of *N. brasiliensis*. Five mice were killed on the day indicated to count worm burden (mean±SE, open bar). EPG (solid circle) was monitored using a group of five mice.

doses of inducing the immunological tolerance, induced a prolongation of infection time. The intestinal worm burdens were high in the treated mice at 7 days PI, when all worms were expelled in the untreated mice (Fig. 2). However, the worm burden in the cyclophosphamide-treated mice decreased at 9 days PI and disappeared within 13 days PI, suggesting a transient prolongation (Fig. 3). No egg was detected in the feces up to 13 days PI in the cyclophosphamide-treated mice.

Cyclophosphamide depletes the cells that are dividing actively *in vivo*, usually B and T lymphocytes. However, its activity is nonspecific. Thus, BALB/c mice were treated with a single injection of 2 mg of anti-CD4 or anti-CD8 antibodies one day before infection and infected with *N. brasiliensis* to see effects of T cell subtypes on the expulsion. Fig. 4 shows that treatment with anti-CD4 but not anti-CD8 antibodies prolonged infection time when estimated at 7 days PI. Although few abnormal eggs were detected per gram feces (150±95, mean ± SE from 4 mice) in the mice treated with anti-CD4 antibody at 7 days PI, it was extremely low compared to EPG in young or nude mice. These results suggest that the short infection of the mouse-nonadaptive *N. brasiliensis* in mice is regulated by $CD4^+$ lymphocytes but fecundity may be controlled by additional factors.

Roles of goblet cells or mucosal mast cells on the

Table 1 Passages of *N. brasiliensis* in young C57BL/6 mice

Exp.	Passages	Age	L_3 infected	Mice egg positive	EPG
1	1	5Wk	600	3/3	16,300±5,300
	2	4Wk	400	2/3	8,500±3,100
	3	4–5Wk	750	2/4	5,500±700
	4	4Wk	470	1/2	11,200
	5	5Wk	500	3/3	4,400±1,800
	6	3Wk	600	0/2	
2	1	4–5Wk	600	2/9	7,600±7,800
	2	5Wk	500	5/6	2,600±1,400
	3	5Wk	500	5/5	6,100±4,300
	4	5Wk	450	2/7	1,300±700
	5	5Wk	120	0/2	

Two to nine young mice of both sexes were infected with larvae obtained from the murine feces of the former passages. For the first passages, larvae from rat's feces were used. EPG (mean±SE) on 7 days PI obtained from the EPG positive mice are shown.

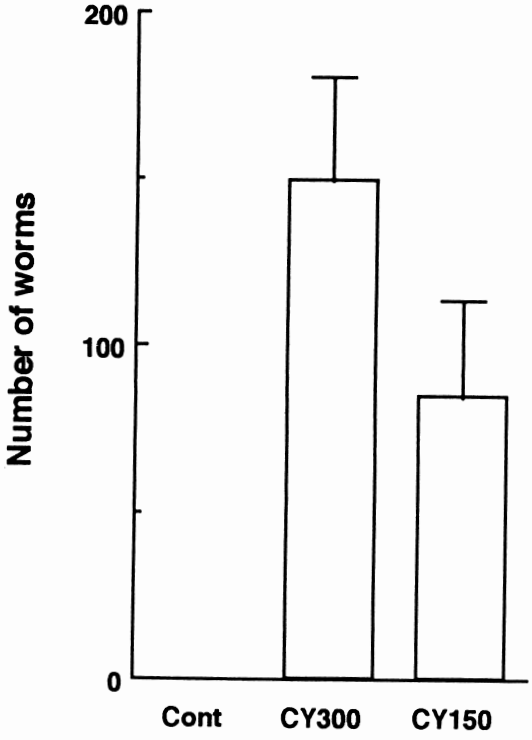

Fig. 2 Effects of cyclophosphamide on intestinal worm recovery. Male BALB/c mice were injected i.p. with 150 or 300 mg/kg of cyclophosphamide (CY) or saline one day before infection and then infected with 400 larvae. Three mice each for cyclophosphamide and 5 mice for saline control were killed on 7 days PI to count intestinal worm burden (mean±SE). P<0.02 in 150 and 300 mg/kg cyclophosphamide vs. control.

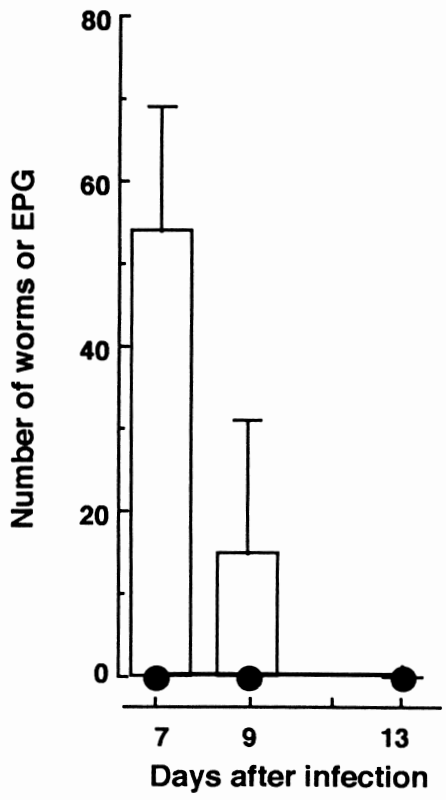

Fig. 3 Kinetics of intestinal worm burden and EPG in BALB/c mice treated with cyclophosphamide. Male mice were injected i.p. with 250 mg/kg of cyclophosphamide one day before infection and then infected with 400 larvae. Four mice were killed on the day indicated to count worm burden (mean±SE, open bar). EPG (solid circle) was monitored using a group of four mice.

spontaneous expulsion of *N. brasiliensis* have been discussed in rats or mice. Therefore, responses of intestinal goblet cells, mucosal mast cells or eosinophils were examined histologically in BALB/c mice infected with the nonadaptive strain of *N. brasiliensis*. Kinetics of goblet cell number was well associated with that of worm burden. Number of goblet cells peaked at 5 days PI with 2.5 times of normal. Number of mucosal mast cells, which was almost zero in normal mice, was markedly increased after 5 days PI and kept increasing until 10 days PI. Number of eosinophils peaked at 7 days PI with 2.9 times of normal. Kinetics of eosinophil number was basically similar to that of goblet cells (Fig. 5).

Discussion

A strain of *N. brasiliensis* used in this study does not deposit eggs in adult BALB/c or C57BL/6 mice, but deposits eggs in athymic nude mice, young mice or rats. We considered first that thymus dependent immune responses protect adult mice from infection with this strain of *N. brasiliensis* and adult worms were not established in their intestines. This study, however, showed that male and female young adult worms but not larvae were recovered from adult mice. When infection time was prolonged few days by cyclophosphamide or anti-CD4 antibody treat-

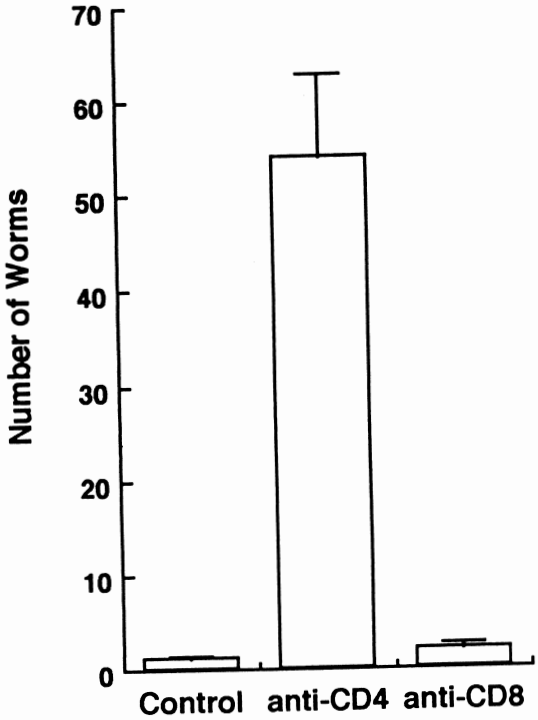

Fig. 4 Effects of anti-CD4 or anti-CD8 antibodies on intestinal worm recovery. Male BALB/c mice were injected i.p. with 2 mg of anti-CD4 or anti-CD8 antibodies, or saline one day before infection and then infected with 400 larvae. Four mice each for antibodies and 5 mice for saline control were killed on 7 days PI to count intestinal worm burden (mean±SE). $P<0.02$ in anti-CD4 treatment vs. control.

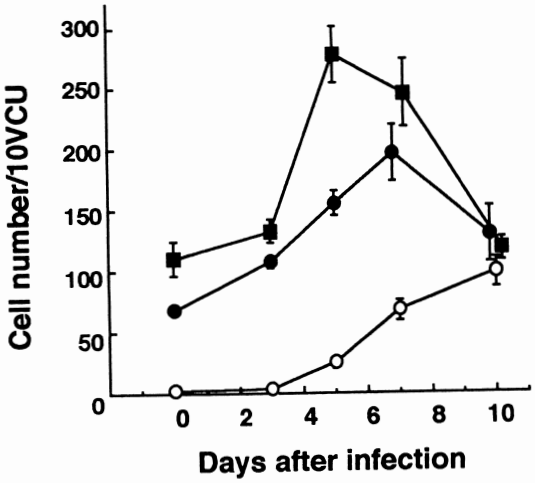

Fig. 5 Kinetics of intestinal goblet cells, eosinophils and mucosal mast cells in *N. brasiliensis*-infection. Male BALB/c mice were infected with 400 larvae and killed on the day indicated. Two sections from a mouse and two to three mice for one point were examined for counting goblet cells (solid square), eosinophils (solid circle) or mucosal mast cells (open circle) per 10 villus crypt unit (VCU).

ments, almost no egg was detected in the feces of treated mice. This prolongation was enough to detect eggs in feces in young mice or athymic nude mice. Therefore, mechanism for inhibiting sexual maturation and that for expelling worms may be different. Thymus dependent mechanisms should be concerned not only with the worm expulsion but also with sexual maturation of the worms. The mechanism for inhibiting the sexual maturation is not clear yet, but is an interesting parasitological question. To study whether the sexual maturation of worms is inhibited irreversibly in mice and whether mouse-adaptive strain can be obtained from this strain of *N. brasiliensis* by more patient experiments may help to answer the question.

As for the worm expulsion, infection time of *N. brasiliensis* is various in mice. Intestinal adult worms of mouse-adoptive strain were recovered in later than 16 days PI in Webster Swiss albino mice (Solomon and Haley, 1966) or at 13 days in BALB/c mice (Urban, Jr. *et al.*, 1993). Obviously the mouse-adaptive strain are expelled slower in mice than the nonadaptive strain used in this study. A strain of *N. brasiliensis* which had been maintained in SD rats and was infected to mice could be recovered from intestines at 10 days PI in CBA/J mice, 14 days PI in C3H/HeJ mice, 8 days PI in A/J mice and 14 days PI in BALB/c mice (Stadnyk *et al.*, 1990). In spite of being maintained in rats, their strain was retained longer than our strain of worms in BALB/c mice. It has not been reported whether their strain of worms deposit eggs in those mice. As far as intestinal worm recovery, their strain seems to be different from the strain of *N. brasiliensis* in this study. For a precise comparison of those differences, microbial contamination of infective larvae or keeping conditions of mice, which may contribute to the host immune responses (Stadnyk *et al.*, 1990), have to be considered.

We showed that $CD4^+$ lymphocytes were important in expelling intestinal *N. brasiliensis* in this study. A similar result has been reported in BALB/c mice with the mouse-adaptive strain (Katona *et al.*, 1988). Effector mechanism against adaptive strain is probably the same as that against nonadaptive strain of *N. brasiliensis* in mice. However, effector mechanism in the expulsion of *N. brasiliensis* is not completely clear yet. Mucosal mast cells induced by recombinant IL-3 injection are important for intestinal protection against *Strongyloides ratti* but not for expulsion of *N. brasiliensis* in mice (Abe *et al.*, 1992, 1993). Little concern with mucosal mast cells in the expulsion of *N. brasiliensis* has also been reported in Mongolian gerbils (Horii and Nawa, 1992; Horii *et al.*, 1993). Mucosal mast cells may be neglected as an important effector cell of the expulsion. Goblet cell hyperplasia or alteration of terminal sugars of goblet cell mucins is important in expulsion of *N. brasiliensis* in rats (Ishikawa *et al.*, 1993). A similar mechanism through goblet cells may be involved in the expulsion in mice. When observed histologically, intestinal goblet cells in the infected mice were large size, showing active secretion of mucus. Number of goblet cells at 5 days PI was increased 2.5 times of normal mice. However, amount of secreted mucus seemed to be more than a change in number. Importance of goblet cells in the expulsion in mice should be studied more in the future. In addition, it is not clear if eosinophils increased on 5 to 7 days PI are concerned with the expulsion and how the $CD4^+$ lymphocytes function is linked to the effector mechanism. Mouse-nonadoptive strain of *N. brasiliensis* is expelled within one week in mice. This system will be a convenient model for studying mechanisms of the expulsion.

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