Journal of Helminthology (2003) **77,** 355–361 DOI: 10.1079/JOH2003187

Strongyloides ratti: the role of interleukin-5 in protection against tissue migrating larvae and intestinal adult worms

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Abstract

To determine the role of interleukin-5 (IL-5) and eosinophils in protection against *Strongyloides ratti*, mice treated with anti-IL-5 monoclonal antibody (mAb) were infected with *S. ratti* larvae. *Strongyloides ratti* egg numbers in faeces (EPG) in mAb treated mice were higher than those in control mice on days 6 and 7 after inoculation. The numbers of migrating worms in mAb treated mice 36 h after inoculation were higher than those observed in control mice. Intestinal worm numbers in mAb treated mice 5 days after inoculation were higher than those in control mice. These results show that eosinophils effectively protected the host against *S. ratti* infection by mainly the larval stage in primary infections. The involvement of eosinophils in protection against secondary infection was also examined. Before secondary infection, mice were treated with anti-IL-5 mAb and infected with *S. ratti*. Patent infections were not observed in either mAb treated or control Ab treated mice. The numbers of migrating worms in the head and lungs of mAb treated mice increased to 60% of that in primary infected mice. Intestinal worms were not found in mAb treated mice or in contcrol mice after oral implantation of adult worms. Eosinophils were therefore mainly involved in protection against tissue migrating worms in secondary infections.

Introduction

Interleukin-5 (IL-5) is a homodimeric glycoprotein and was initially identified by its ability to support the growth and differentiation of B cells (Takatsu, 1998). Interleukin-5 has been shown to possess various biological activities in different cell types, including B cells, T cells and eosinophils. Interleukin-5 selectively promotes the growth and differentiation of eosinophils (Hitoshi *et al.*, 1991) and eosinophilia (increased numbers of eosinophils in peripheral blood or tissue) is IL-5 dependent, *in vivo* (Coffman *et al.*, 1989).

Eosinophilia is a characteristic of the host response during parasitic helminth infections (Behm & Ovington, 2000; Meeusen & Balic, 2000). Eosinophils are involved in immunity against various parasites (Butterworth *et al.*,

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1975; Shin *et al.*, 1997; Sugaya *et al.*, 1997), including *Strongyloides* species (Korenaga *et al.*, 1991; Ovington *et al.*, 1998; Herbert *et al.*, 2000).

Strongyloides ratti is a parasitic nematode found in the intestine of rodents. Infective larvae penetrate the host's skin and migrate mainly to the head through the connective tissue of the subdermis. Larvae then migrate to the host's gastrointestinal tract and moult twice to become adult worms in the upper intestine (Tada *et al.*, 1979). During infection, several host protective responses occur. For example, serum IgE concentrations increase (Watanabe *et al.*, 2001) and the number of mast and goblet cells increases in the intestine, where mast cells have been shown to act as effector cells against *S. ratti* and *S. venezuelensis* (Nawa *et al.*, 1985; Maruyama *et al.*, 2000).

The aim of this study was to investigate the role of IL-5 and eosinophils in protecting against *S. ratti* by using anti-IL-5 monoclonal antibody (mAb), and taking the *S. ratti* life-cycle into consideration. For this purpose, the effects of the mAb treatment on the murine immune system were assessed by counting migrating worms (larvae) and intestinal worms (adult) separately.

Materials and methods

Animals

Male C57BL/6 mice (6–8 weeks of age) and female Wistar rats (8 weeks of age) were purchased from Japan SLC Inc., Shizuoka, Japan. All animal experiments were performed under specific pathogen-free conditions. All experiments were conducted in accordance with the principles and procedures outlined in the Guidelines for the Care and Use of Laboratory Animals in Nagasaki University.

Parasite and parasitological techniques

Strongyloides ratti, TMDU strain, was maintained in our laboratory by serial passage in Wistar rats. Infective larvae (L3) were obtained by the filter-paper culture of faeces from infected Wistar rats (Tada *et al.*, 1979). Before infection, larvae were washed several times with sterile saline and then with saline containing 200 IU ml^{-1} penicillin and 200 $\mu g\,ml^{-1}$ streptomycin. Finally, larvae were resuspended in sterile saline and 0.2 ml of suspension containing 1000 or 2000 L3 were injected subcutaneously into mice. Larval counts in the head and lungs or adult worm counts in the small intestine were done as described previously (Watanabe *et al.*, 1999). Counts of eggs or larval excretions into the faeces (EPG) were performed as described previously (Watanabe *et al.*, 2000).

Monoclonal rat anti-mouse IL-5 antibody (anti IL-5 mAb)

Anti-mouse IL-5 antibody (IgG1 subclass) was obtained from the hybridoma, TRFK-5, which was kindly donated by Dr R.L. Coffman, DNAX, Palo Alto, California, USA. Normal rat serum which was treated with saturated ammonium sulphate (pH 7.2) precipitation and dialysed against PBS was used as the rat immunoglobulin (Ig) control. Protein concentration was determined by Protein Assay® (Bio-Rad Lab., Richmond, California, USA). Aliquots were kept at −20 °C until use.

Experimental design

To investigate the role of eosinophils in protecting against *S. ratti* primary infection, eggs in the faeces (EPG), and the numbers of migrating larvae and intestinal adult worms were assessed in *S. ratti* infected mice. There were two groups of mice. One group which comprised four mice was treated with 2 mg of rat Ig every 7 days from 14 days before infection to 14 days after inoculation. The other group which comprised five mice was treated with 2 mg of anti-IL-5 mAb on the same days as rat Ig treatment. Two groups of mice were infected with 2000 L3 of *S. ratti* on day 0. To count adult worm numbers in the intestine, mice were sacrificed 5 days after inoculation. To count migrating larvae in the head and lungs, another two groups of mice treated as described above were infected with 1000 *S. ratti* L3. These mice were sacrificed 36 h after inoculation and migrated worms in the head and lungs were counted.

In order to investigate the role of eosinophils against *S. ratti* secondary infection, the EPG, number of migrating larvae and of intestinal adult worms were assessed in secondary infected mice. There were three groups of mice. The first group, which comprised five mice, was infected with 2000 L3 on day 0 and treated with 2 mg of rat Ig on day 21 and 27, then challenged with 2000 L3 on day 27. The second group, which comprised five mice, was infected with 2000 L3 on day 0 and treated with 2 mg of anti-IL-5 antibody on the same day as rat Ig treatment and then challenged with 2000 L3 on day 27. The primary infection control group which comprised four mice was not infected with *S. ratti* on day 0, but was subsequently treated with rat Igs on days 21 and 27; these mice were infected with 2000 L3 on day 27. These three groups of mice were sacrificed 36 h after challenge inoculation, and migrating worms in the head and lungs were recovered.

To examine mast cell activation and adult worm numbers in the intestine, 400 adult worms recovered from infected Wister rats were orally implanted into three groups of mice treated as above. Mice were sacrificed 19 h after implantation and adult worm numbers were counted.

Serum mouse mast cell protease-1 (MMCP-1) enzyme-linked immunosorbent assay (ELISA)

There are several reports showing that mast cells are essential for the protection against *Strongyloides* adult worms (Nawa *et al.*, 1985; Maruyama *et al.*, 2000). Mouse mast cell protease-1 is released systematically during mucosal mast cell activation and worm expulsion (Newlands *et al.*, 1987; Onah *et al.*, 2000). In order to examine the relationship between mast cell activation and adult worm expulsion, adult worms recovered from infected rat intestines were orally implanted into mice treated as described above, on day 27 after primary infection. Worm numbers and MMCP-1 concentrations were measured 19 h after oral implantation of adult worms. Serum MMCP-1 concentrations were measured using a commercial MMCP-1 ELISA kit (Moredun Scientific Ltd, Edinburgh, UK), according to the

https://doi.org/10.1079/JOH2003187 Published online by Cambridge University Press

manufacturer's instructions. Briefly, serially diluted serum (1:10–10000) samples were loaded on a 96-well ELISA plate coated with 2 μg ml^{-1} sheep anti MMCP-1 capture antibody. The plate was incubated for 1.5 h at room temperature. Then the plate was washed with PBS–Tween-20 five times, and incubated with rabbit anti MMCP-1 antibody conjugated with horseradish peroxidase for 1 h. After washing the plate as before, orthophenylene diamine (OPD) (WAKO Chemicals, Japan) in citrate phosphate buffer was added to the wells. The reaction was stopped by the addition of 2.5 N H_2SO_4 and the plate read at 492 nm.

Peripheral blood eosinophil counts and differentials

Peripheral blood eosinophils were counted using an improved Neubauer haemocytometer after staining with Hinkelman's solution (0.5% w/v eosin Y, 0.5% w/v phenol and 0.185% v/v formaldehyde in distilled water). Peripheral blood differential counts were determined on blood smears after May-Grünwald Giemsa staining.

Statistics

Differences between the Ig treated control and anti-IL-5 mAb treated mice were determined by Student's t-test. In the case of eosinophil numbers, the statistical significance was calculated using the Mann-Whitney U-test as eosinophil numbers in anti-IL-5 mAb treated mice were not found to follow a normal distribution.

Results

Eosinophil depletion by anti-IL-5 mAb (TRFK-5) treatment and primary infection in anti-IL-5 mAb treated mice

Mice were treated with 2 mg of anti-IL-5 mAb every 7 days from 14 days before infection to 14 days after infection. Treatment from 14 days before infection effectively reduced eosinophil numbers at the time of infection (fig. 1) and continuously suppressed the eosinophilia during the course of infection.

The EPG in anti-IL-5 mAb treated mice infected with 2000 *S. ratti* L3 was significantly (2–3 times) higher than those in control mice on days 6 and 7 post-infection (fig. 2). After this time, EPG in both mice decreased to be almost nil on day 12 post-infection. The duration of the primary infection did not differ between control and treated mice.

Numbers of migrating larvae in the head and lungs, and of adult worms in the intestine in anti-IL-5 mAb treated mice

The number of infective larvae used for infection was reduced to 1000 in order to make the counts more accurate. The results of this experiment are shown in table 1. The numbers of migrating larvae in the head in anti-IL-5 mAb treated mice 36 h after inoculation were 1.5 times higher than those in control mice. We have shown previously that monocytes and granulocytes (especially neutrophils) are involved in the protection against migrating larvae (Watanabe *et al.*, 2000); therefore, differential peripheral blood counts in mAb treated mice were also determined at the time of infection.

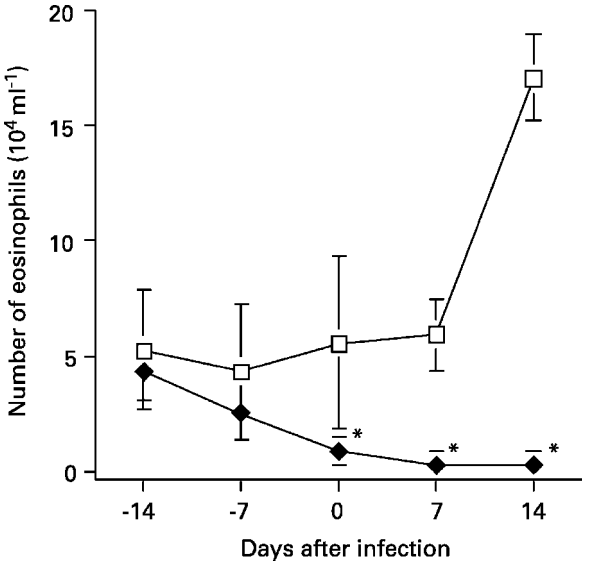

Fig. 1. Kinetics of eosinophil numbers in anti-IL-5 mAb treated mice during primary infection of *Strongyloides ratti*. Each data point represents the mean ± SD of 4–5 mice; □, control mice; ◆, anti-IL-5 mAb treated mice. *$P <$ 0.05 when compared with control mice.

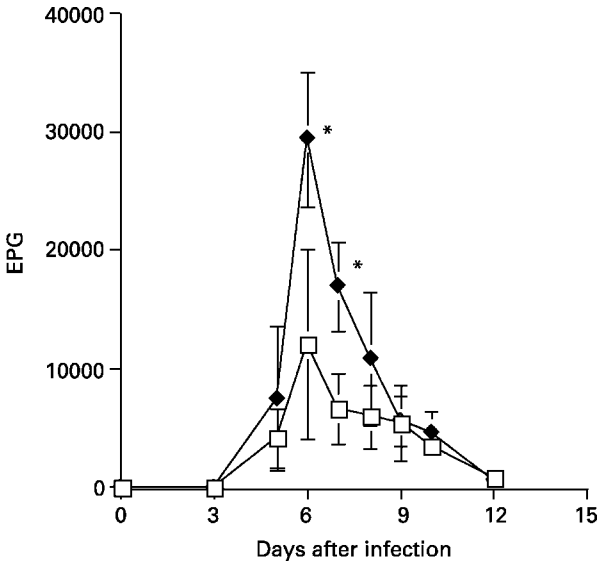

Fig. 2. Numbers of parasite eggs per gram of faeces (EPG) from anti-IL-5 mAb treated mice during primary infection of *Strongyloides ratti*. Each data point represents the mean ± SD of 4–5 mice; □, control mice; ◆, anti-IL-5 mAb treated mice. *$P < 0.05$ when compared with control mice.

Anti-IL-5 mAb treatment did not alter the proportion of any cells except for eosinophils.

The number of adult worms in the intestine in anti-IL-5 mAb treated mice was twice that in control mice 5 days after infection.

Therefore, these results suggest that eosinophils have a protective function against migrating larvae of *S. ratti*, even in a primary infection.

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Table 1. Effect of anti-IL-5 mAb treatment on peripheral blood cells and numbers of migrating larvae and adult worms in mice infected with *Strongyloides ratti*.

	Peripheral blood differential counts (%) at the time of infection§				Number of migrating larvae§		Number of adult worms in intestine¶
	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Head	Lung	
Rat Ig group	8.1 ± 1.6	84.1 ± 3.7	5.5 ± 1.1	3.0 ± 0.5	95.2 ± 28.7	20.0 ± 9.2	108.3 ± 34.9
Anti-IL-5 mAb group	6.7 ± 1.9	86.9 ± 1.8	6.1 ± 1.3	0.3 ± 0.5*	152.3 ± 39.4**	19.2 ± 6.1	200.2 ± 20.2**

§ Mice were treated as described in the text and infected with 1000 *S. ratti* L3. Thirty-six hours after inoculation mice were sacrificed and migrated worms were recovered.
¶ Mice were treated as described in the text and infected with 2000 *S. ratti* L3. Five days after inoculation mice were sacrificed and worms in the intestine were recovered.
* $P < 0.05$ compared with rat Ig group.
** $P < 0.05$ compared with rat Ig group.
Each value represents the mean ± SD of 4–5 mice.

Eosinophil depletion by anti-IL-5 mAb treatment in secondary infection

Mice were treated with anti-IL-5 mAb on days 21 and 27 after primary infection and subjected to a challenge infection with 2000 *S. ratti* L3 on day 27. Mice were also treated with the antibody on day 34 after primary infection. Anti-IL-5 treatment effectively reduced eosinophil numbers in the secondary infection (fig. 3). This treatment continuously suppressed the eosinophil numbers during the experiment. Without antibody treatment, eosinophil numbers in control mice increased markedly.

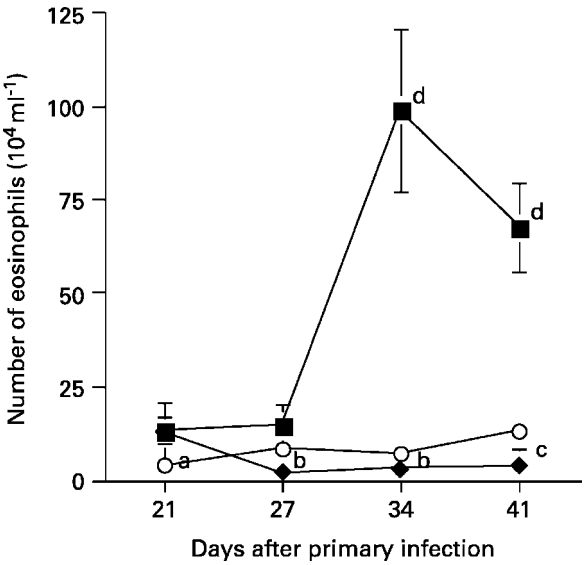

Fig. 3. Kinetics of eosinophil numbers in anti-IL-5 treated mice during secondary infection of *Strongyloides ratti*. Each data point represents the mean ± SD of 4–5 mice. ■, Rat Ig treated secondary infected mice; ◆, anti-IL-5 mAb treated secondary infected mice; ○, rat Ig treated primary infection control group. [a]$P < 0.05$ when compared with secondary infected mice (rat Ig treated or anti-IL-5 mAb treated). [b]$P < 0.05$ when compared with rat Ig treated secondary infected mice. [c]$P < 0.05$ when compared with rat Ig treated secondary infected mice, or rat Ig treated primary infected mice. [d]$P < 0.05$ when compared with anti-IL-5 mAb treated secondary infected mice or rat Ig treated primary infected mice.

Secondary infection was monitored by EPG. No patent infections were observed in both anti-IL-5 antibody treated mice and control mice (data not shown).

Numbers of migrating worms in the head and lungs, and adult worms in the intestine in anti-IL-5 mAb treated mice during secondary infection

In anti-IL-5 mAb treated mice (anti-IL-5 mAb secondary infection group), the number of migrating worms 36 h after secondary infection increased to 60% of those in primary infected rat Ig treated mice (rat Ig primary infection group), whereas, in rat Ig treated and secondary infected mice (rat Ig secondary infection group), few worms were recovered in the head and lungs (table 2).

No worms were recovered from the intestine 5 days after secondary infection in either control or treated mice (data not shown).

Serum mouse mast cell protease-1 (MMCP-1) in secondary infected mice after oral implantation of adult worms

To evaluate the relationship between the mast cell activation in the intestine and adult worm establishment, MMCP-1 concentrations were measured after adult worm implantation. Regardless of the anti-IL-5 antibody treatment, mice subjected to secondary infection by oral implantation with adult worms showed a high concentration of MMCP-1 in the serum, which meant that mucosal mast cells had been activated (table 3). In accordance with high MMCP-1 concentrations, no adult worms were found in the secondary infected mice even 19 h after inoculation.

Discussion

The present study clearly indicates that eosinophils are involved in protection against *S. ratti*, in both primary and secondary infections. Repeated injection of anti-IL-5 mAb into non-infected mice effectively reduced eosinophil numbers (fig. 1). In primary infections, numbers of migrating larvae and of intestinal adult

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Table 2. Recovery of migrating larvae in the head and lung of anti-IL-5 mAb treated mice challenged with *Strongyloides ratti*.

Group	Primary inoculation Day 0	Anti-IL-5 mAb treatment Day 21	Anti-IL-5 mAb treatment Day 27	Secondary inoculation Day 27	Eosinophils number (10^4 ml^{-1}) Day 21	Eosinophils number (10^4 ml^{-1}) Day 27	Migrating larvae number§ Head	Migrating larvae number§ Lung
Rat Ig secondary infection	2000 L3	(−)	(−)	2000 L3	13.5 ± 6.9	9.4 ± 2.2	18.5 ± 15.3	22.3 ± 19.0
Anti-IL-5 mAb secondary infection	2000 L3	(+)	(+)¶	2000 L3	18.8 ± 8.1	2.2 ± 1.9[b]	147.2 ± 61.5[c]	55.0 ± 15.9[c]
Rat Ig primary infection	(−)	(−)	(−)	2000 L3	4.3 ± 1.1[a]	7.8 ± 1.9	242.8 ± 43.4[d]	87.0 ± 11.1[d]

§ Mice were sacrificed 36 h after challenge (secondary) infection and migrating larvae were recovered.
¶ Mice were treated with anti-IL-5 mAb 2 h before challenge.
[a] $P < 0.05$ when compared with rat Ig secondary infection group or anti-IL-5 mAb secondary infection group.
[b] $P < 0.05$ when compared with rat Ig secondary infection group or rat Ig primary infection group.
[c] $P < 0.05$ when compared with rat Ig secondary infection group or rat Ig primary infection group.
[d] $P < 0.05$ when compared with rat Ig secondary infection group or anti-IL-5 mAb secondary infection group.
Each value represents the mean ± SD of 4–5 mice.

Table 3. Intestinal worm recoveries and serum MMCP-1 concentrations 19 h after oral implantation of 400 *Strongyloides ratti* adult worms.

Group	Recovered worm numbers	MMCP-1 concentration (ng ml^{-1})
Rat Ig secondary infection§	0 ± 0	2419 ± 980**
Anti-IL-5 mAb secondary infection§	0 ± 0	2444 ± 1992**
Rat Ig primary infection§	18.3 ± 8.3*	7.1 ± 1.1

§ Mice were treated as table 2 and then implanted with 400 adult worms 2 h after second anti-IL-5 mAb treatment or rat Ig. Mice were sacrificed 19 h after implantation and intestinal worm numbers were counted.
* $P < 0.05$ when compared with rat Ig secondary infection or anti-IL-5 secondary infection group.
** $P < 0.05$ when compared with rat Ig primary infection group.
Each value represents the mean ± SD of 4–5 mice.

worms in anti-IL-5 mAb treated mice were significantly higher than those in control mice (table 1). As the numbers of migrating larvae were counted only 36 h after inoculation, the eosinophils that protected against tissue migrating larvae may be circulating eosinophils and recruited into the tissues around the larvae. Circulating or pooled eosinophils can be recruited within several hours of eotaxin injection (Mould *et al.*, 1997). An increase in circulating eosinophils was observed at day 14 post-infection but these eosinophils were probably not associated with host immunity against the primary infection.

Anti-IL-5 mAb treatment in mice during a primary infection increased the migrating worms recovery only in the head (table 1). In our previous report (Watanabe *et al.* 2000), the same phenomenon was observed in anti-granulocytes mAb treated mice. One possibility is that a major portion of the *S. ratti* infective larvae migrated to the head through the connective tissue and the remainder migrated to the lungs (Tada *et al.*, 1979). Almost all circulating eosinophils accumulated around the connective tissue migrating larvae, whereas few cells accumulated around the worms migrating to the lungs, under normal conditions. Therefore, abrogation of eosinophils did not affect the number of migrated worms in the lungs. Dawkins *et al*. (1981) observed the lesion histopathologically and reported that larvae in the skin were surrounded by neutrophils and eosinophils 24 h after infection and a few worms showed signs of lysis. In the lung, larvae lay in the alveolae and no inflammatory cells were present. This work of Dawkins *et al.* (1981) supports our hypothesis. The fact that larvae chiefly migrate to the head through the subdermis and no inflammation occurs around the larvae in the lung indicates that the subdermal connective tissue is the site of host protection against *S. ratti* migrating larvae in primary infection. To estimate the number of migrating larvae in the head is to estimate., the host defence against *S. ratti* migrating larvae in primary infection.

The intestinal worm number and EPG in anti-IL-5 mAb treated mice also increased on days 6 and 7 (table 1, fig. 1). The number of migrating larvae has already increased in the mAb treated mice 36 h after infection. Therefore the observed increase in the number of adult worms in the intestine may be related to an increased number of migrating larvae.

The duration of primary infection was not affected by the antibody treatment, which suggested that eosinophils did not contribute to host expulsion of the adult worms from the intestine. Mast cells, and their granular content, have shown to be essential for the host to expel the adult worm (Nawa *et al.*, 1985; Maruyama *et al.*, 2000).

In a secondary infection of *S. ratti*, eosinophil numbers dramatically increased and this eosinophilia was suppressed by anti-IL-5 mAb treatment (fig. 3).

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In accordance with suppressed eosinophil numbers, the number of migrating worms was recoverable (table 2). At the time of infection, eosinophil numbers had already been raised by the primary infection and increased eosinophils protected the host from the secondary infection.

We did not find any patent infection after secondary infection with *S. ratti* in anti-IL-5 mAb treated mice in which migrating larvae were recovered. Adult worms could not establish in the intestine because of immunity from the primary infection (table 3). Several reports have shown that intestinal mucosal mast cells are an effective defence against intestinal worms of *S. ratti* and *S. venezuelensis* (Nawa *et al.*, 1985; Abe *et al.*, 1993; Maruyama *et al.*, 2000). The present study supports the association between worm expulsion and mast cell activation (table 3). Anti-IL-5 treatment did not affect the mast cell activation, which was already induced by primary infection.

There have been many reports concerning the relationship between eosinophils and helminth infections (Behm & Ovington, 2000; Meeusen & Balic, 2000). In the case of *S. ratti*, Ovington *et al*. (1998) reported that increased worm recovery and EPG were observed in IL-5 knockout mice (Ovington *et al.*, 1998). The present work is consistent with this report except for the number of migrating worms recovered in secondary infections. Taking these results into consideration, we propose that eosinophils have a protective role against tissue migrating worms in both primary and secondary infections.

Acknowledgements

We are grateful to Dr R. L. Coffman, DNAX Research Institute of Molecular and Cellular Biology, for providing the TRFK-5 hybridoma cell line. This work was supported in part by Grants-in-Aid (08457081, 13770126) for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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(Accepted 7 April 2003)