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Inhibition of Eosinophil Infiltration and Humoral Immune Reaction by Ketotifen in BALB/c Mice Infected with *Echinostoma hortense*

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Eosinophils play an essential role in allergy reaction after parasite infection. To examine the immune reaction induced by eosinophils, we investigated the allergy reaction in BALB/c mice infected with *Echinostoma hortense's* metacercariae, as well as the effect of ketotifen, an anti-allergy drug, on eosinophil immune reaction in the villi of host intestine. The worm recovery rate was higher in ketotifen-treated mice than in untreated mice and the worms in ketotifen-treated mice survived longer than those in untreated mice. The antibody titer in the serum of ketotifen-treated mice was very low. Especially, *Echinostoma hortense* infection strongly increased serum IgE level and eosinophil infiltration into the villi of the mouse intestine. Ketotifen treatment suppressed eosinophil infiltration into the infected areas and inhibited IL-4 production. The reduced IL-4 production may be related with the reduction of IgE, IgG1 and IgG2 production. In conclusion, ketotifen inhibited eosinophil infiltration functioning in the allergy reaction induced by parasite infection and the expression of immunoglobulins and cytokines.

Key Words: *Echinostoma hortense*, Ketotifen, Eosinophils, BALB/c mice

INTRODUCTION

After a parasite infects a host, it matures and then settles. Some infected parasites are inhibited by host immune response and they are excreted after an infection period (Chai, et al., 1985; Chai, et al., 1985; Wakelin, et al., 1993). An allergy infection is known as a specific immune response against various parasite infections. Eosinophil infiltration happens in the villi of a host intestine and serum IgE titer increases. IgE-mediated type I allergy reaction is associated with the production of cell affinity antibody (Ishizaka, et al., 1976; Jarrett, et al., 1974; Kingger, 1997; Kojima, et al., 1972; Rihet, et al., 1991; Rouseaux-Prevost, et al., 1979; Saito, et al., 1996).

An infection of intestine flukes induces immune responses, including eosinophil infiltration into tissues and serum IgE level (Dessaint, et al., 1975; Hogarth-Scott, et al., 1969; Ito, et al., 1976; Juhlin, et al., 1969). Cytokine plays a key role in the immune response (Callard, et al., 1990; Calvert, et al., 1990; King, et al., 1990; Lagente, et al., 1995; Phillps, et al., 1990).

Ketotifen has been known as an anti-allergy drug and it regulates a variety of enzymes in the cell membrane and histamine receptor functioning in immunomodulation (Grant, et al., 1990). In addition, ketotifen increases beta receptor expression and inhibits secreted mediators (Castillo, et al., 1991; Cordoba, et al., 1992). Inhibition of IgA secretion due to ketotifen treatment suppresses lymphocyte proliferation, resulting in IgG1 and IgG2 down-regulation (Doligalska, et

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al., 2000; Kondo, et al., 1994). Ketotifen decreases IL-4 expression associated with eosinophil activation, IgE production, and antigen absorption. Ketotifen decreases serum Th2 cytokine and reduces IgA, PEG2, and LTB. Ketotifen has been used in asthma and allergy therapy because it enhances nitric oxide synthase (NOS) activity (Eliakim, et al., 1992; Kameli, et al., 1991; Konno, et al., 1994).

In the present study, we investigated eosinophil infiltration in BALB/c mice infected with *Echinostoma hortense's* metacercariae and explored the anti-allergy effect induced by ketotifen. To examine the alteration of immune response due to ketotifen, serum IgA, IgE, IgG1, IgG2 and cytokine (IFN-γ, IL-12, IL-4) levels were monitored by ELISA.

MATERIALS AND METHODS

1. Experimental animals

Six-week old, female BALB/c mice were obtained from the Korean Experimental Animal Center. There were 24 mice in both the untreated and ketotifen-treated groups and they were monitored for 8 weeks.

2. Ketotifen treatment and metacercaria infection

The ketotifen group was fed with ketotifen (0.1 mg/kg) via a gavage needle one week before metacercaria infection. *E. hortense* metacercariae were collected by an artificial digestion of *Misgurnus anguillicaudatus* caught in Munmak, Kangwondo, Korea. Thirty metacercariae underwent oral challenge.

3. Worm recovery rate

After metacercaria infection, three mice in each group were anaesthetized with ethyl ether and sacrificed at one-week intervals. The intestine was cut and incubated in saline solution for 2 hours. Adult worms were collected and counted. The worm recovery rate was determined as the number of metacercariae/number of recovered adult worms × 100.

4. Lendrum's method

The duodenum, jejunum, and ileum from the small intestine were divided, washed, and fixed with Carnoy's solution at 4℃. After mounting on paraffin block, fixed tissues were stained with Mayer's hematoxylin for nucleus identification. The samples were washed, stained with Carbol-chromotrope solution, and mounted with Canada balsam.

5. Counting of eosinophils

The number of eosinophils was calculated as previously described (Wakelin, et al., 1993). Ten villi were counted in each region of intestine and all counts were expressed as the number of cells per villus-crypt unit (VCU).

6. ELISA

The presence of serum IgG1, IgG2a, and IgE isotypes specific to *E. hortense* was determined by ELISA as previously described (Voller, et al., 1976). In addition, the Sandwich method was performed to detect the presence of cytokines, IFN-γ, IL-4 and IL-12 in serum. The antigen of *Echinostoma hortense* was diluted to 5 μg/ml in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and used to coat the wells of a polystyrene microplate. After 1 hr incubation at 37℃, plates were washed three times with PBST (Phosphate-buffered saline [pH 7.0] with 0.05% Tween 20). For blocking non-specific binding, plates were incubated with 3% BSA (Bovine serum albumin, sigma) in PBST for 1 hr at 37℃ and then incubated with an appropriate dilution of the sera for 2 hr at 37℃ and washed three times. Isotype-specific, anti-mouse horseradish peroxidase conjugates (Serotec, Kidlington, United Kingdom) were added (100 μl/well) at 1:1000 or 1:2000 dilution. After 1 hr incubation at 37℃, plates were washed three times, and 100 μl of substrate solution (*o*-phenylenediamine 0.5 mg/50 ml, 0.006% H_2O_2) was added to each well. After 1 hr incubation in the dark, the enzyme reaction was stopped by

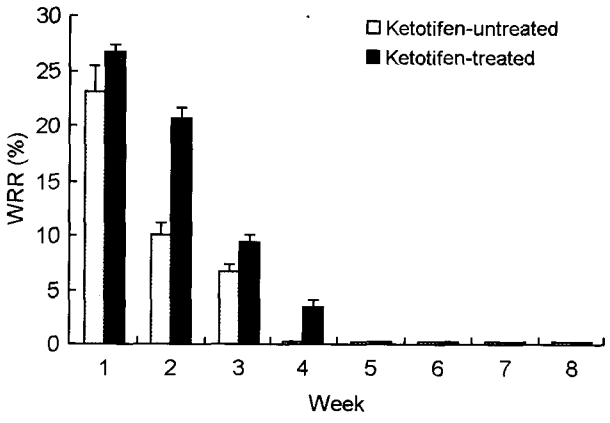

Fig. 1. Worm recovery rate (%) of *E. hortense*-infected BALB/c mice with ketotifen treatment.

addition of 25 μl of 2.5 M H_2SO_4, and the absorbance of the developed color was measured at 490 nm with an automatic microplate reader (Molecular Devices, Sunnyvale, CA, USA).

RESULTS

1. Ketotifen treatment and worm recovery rate

We firstly examined whether ketotifen affects the worm

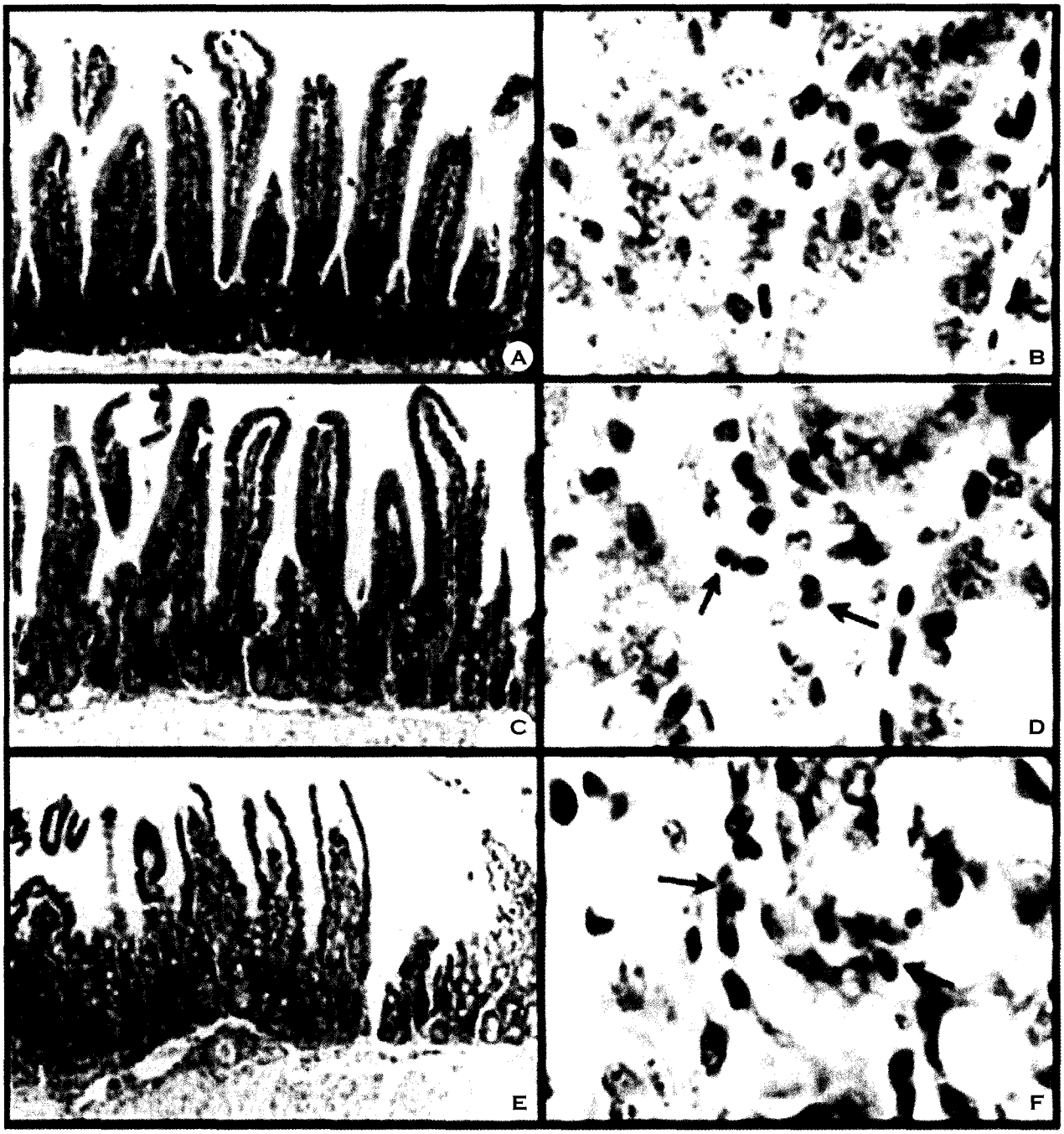

Fig. 2. Microphotographs of intestinal duodenum villi showing eosinophils in BALB/c mice. Lendrum's staining was performed to identify eosinophils in duodenum villi. **A** and **B,** *E. hortense*-non-infected experimental controls; **C** and **D,** *E. hortense* infection and ketotifen-untreated; **E** and **F,** *E. hortense*-infected and ketotifen treatment. Original magnifications: **A, C** and **E,** × 100; **B, D** and **F,** × 1000. Arrows indicate the eosinophils.

recovery rate in BALB/c mice infected with *E. hortense*. As shown in Fig. 1, the worm recovery rate in the BALB/c mice decreased after week 2 post-infection (P.I.) and disappeared at week 4 P.I. (Ed- note that because your measurements are at discrete, rather than continuous, time points of each week, the appropriate preposition is 'at' rather than 'in' the week) Ketotifen treatment increased both the worm recovery rate at weeks 1 and 2 P.I. and the total period of worm recovery (Fig. 1).

2. Eosinophils in intestinal mucosa

Eosinophils in the *E. hortense*-infected mice were affected by ketotifen treatment. The number of eosinophils in the ketotifen-treated group at weeks 2, 4, 6, and 8 P.I. was 5.3±0.5, 16.7±1.5, 10.7±1.1, and 4.7±0.5, respectively (Fig. 2). The number of eosinophils in the control group was 8.3±1.1, 20.7±2.5, 18.7±1.1, and 12.7±1.1, respectively, indicating that ketotifen decreased the number of eosinophils. In the intestinal villi, the number of eosinophils reached a peak at week 4 P.I. and then rapidly declined (Fig. 3).

3. Alteration of antibodies induced by ketotifen treatment

Serum IgA in the control group and the ketotifen-treated group reached a peak at week 7 P.I. However, absorbances in serum IgA of the control and ketotifen groups were 0.45±0.03 and 0.21±0.03, respectively (Fig. 4). Serum IgE in the control and ketotifen-treated groups reached a peak at week 7 P.I. However, absorbances in serum IgA of the control group and the ketotifen-treated group were 0.45±0.03

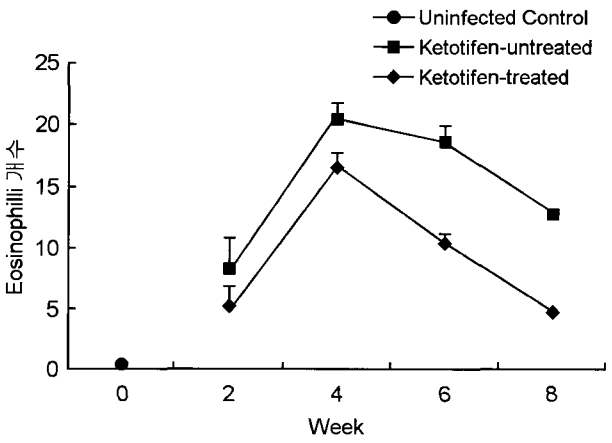

Fig. 3. Average number of eosinophils in duodenum of ketotifen-treated BALB/c mice

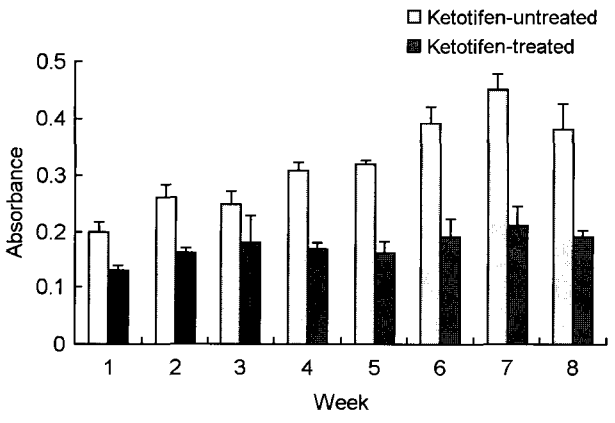

Fig. 4. IgA change in the serum of BALB/c mice infected with *E. hortense* by ketotifen treatment.

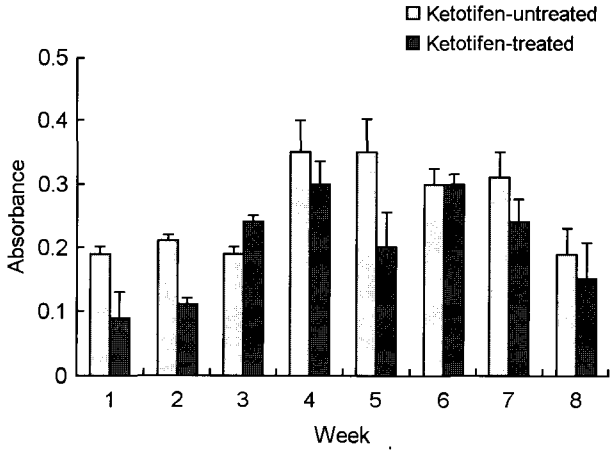

Fig. 5. IgE change in the serum of BALB/c mice infected with *E. hortense* by ketotifen treatment.

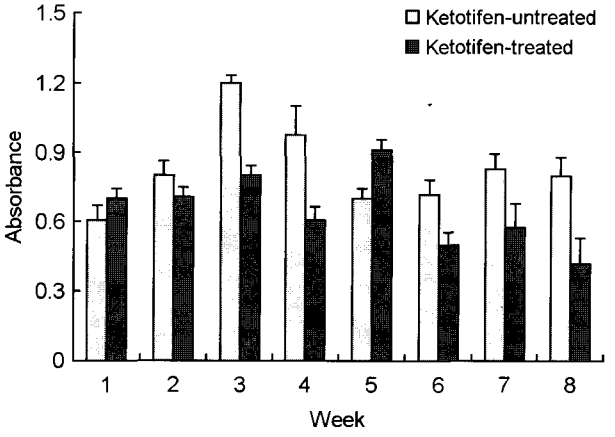

Fig. 6. IgG1 change in the serum of BALB/c mice infected with *E. hortense* by ketotifen treatment.

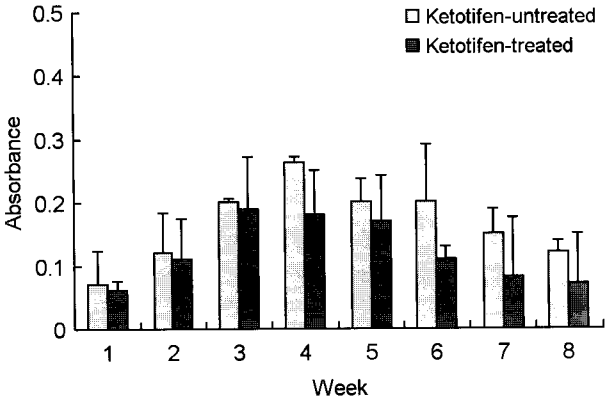

Fig. 7. IgG2 change in the serum of BALB/c mice infected with *E. hortense* by ketotifen treatment.

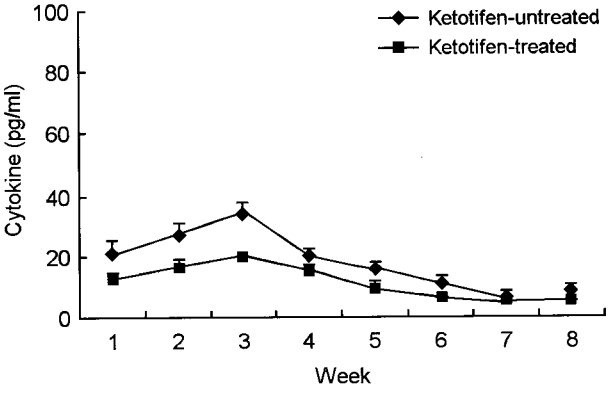

Fig. 9. IL-12 change in the serum of BALB/c mice infected with *E. hortense* by ketotifen treatment.

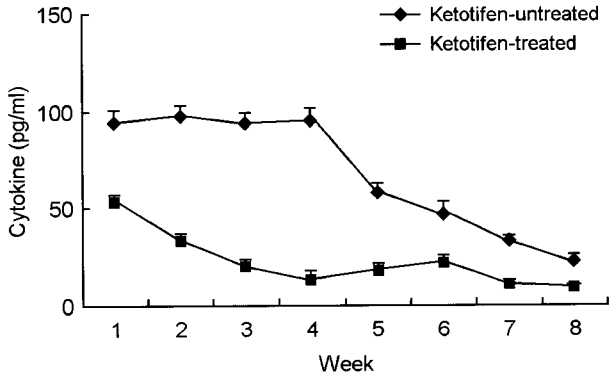

Fig. 8. IFN-γ change in the serum of BALB/c mice infected with *E. hortense* by ketotifen treatment.

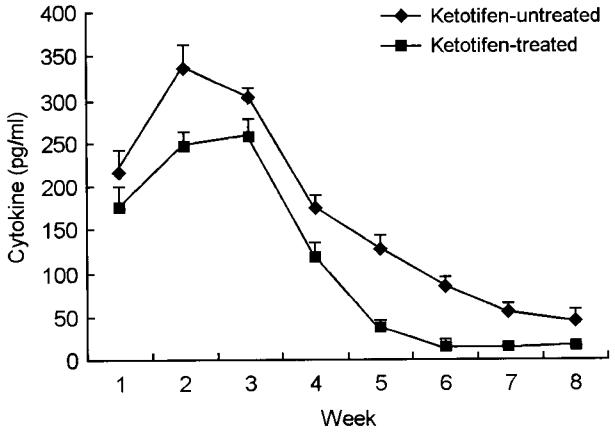

Fig. 10. IL-4 change in the serum of BALB/c mice infected with *E. hortense* by ketotifen treatment.

and 0.21±0.03, respectively (Fig. 4). Absorbance in serum IgE of the control group was 0.35±0.5 at weeks 4 and 5 P.I. Serum IgE in the ketotifen group was lower than in the control group (Fig. 5). Absorbance in IgG1 in the control group gradually increased, peaked at week 4, and then gradually decreased (Fig. 6). IgG1 in the ketotifen-treated group peaked in week 5 (0.9±0.04) and then weakly lessened. IgG1 showed higher antibody titer than the other antibodies. Absorbance in serum IgG2 of the control group was 0.26±0.01 at week 4 P.I. However, Serum IgG2 in the ketotifen group peaked at week 3 P.I. (0.19±0.08) and was lower than in the control group (Fig. 7).

4. Cytokine production

IFN-γ, known as Th1 cytokine, in the control group maintained a constant level after infection and decreased after week 5 P.I. However, IFN-γ in the ketotifen group gradually decreased from week 1 P.I. to week 4 P.I. (Fig. 8). IFN-γ in the ketotifen group was lower than in the control group. Since IL-12 production was very low, we could not find any difference between the control and ketotifen groups in alteration of IL-12 concentration (Fig. 9). IL-4, included in the Th2 cytokine family, in the control group showed a peak level at week 3 P.I., while the ketotifen-treated group showed lower IL-4 production than the control group (Fig. 10).

DISCUSSION

To investigate immune responses in the intestinal mucosa of a host after parasite infection, we examined worm recovery rate, eosinophil infiltration, and cytokine production in mice infected with *E. hortense* metacercariae. In addition, we examined whether immune response was affected by

ketotifen, which is known as a drug in asthma and allergy therapy, and as an inhibitor of cytokine production (Eliakim, et al., 1992; Kameli, et al., 1991; Konno, et al., 1994).

It is known that parasites are affected by immune responses (Chai, et al., 1984; Wakelin, et al., 1993). IgE level increases after various parasite infections, including *Ascaris lumbricoides*, *Toxocara canis*, *Schistosoma japonicum*, *Wuchereria bancroft*, *Schistosoma mansoni*, and *Schistosoma haematobium* (Hogarth-Scott, et al., 1969; Ito, et al., 1976; Juhlin, et al., 1969; Kojima, et al., 1972). Experimental animals infected with a parasite show IgE upregulation (Rouseaux-Prevost, et al., 1979).

Fischel et al. (1952) and Germuth et al. (1952) reported that corticosteroids, acting as an immunosuppressor, increased parasite infection in mice. Chai et al. (1984) reported that prednisolone increased worm recovery rate and infection time after *Metagonimus yokogawai* infection. Ito and Kamiyama (1987) reported that cortisone increased the number of eggs in nude mice infected with *Hymenolepis nana*. Dexamethasone inhibited worm recovery rate in *Echinostoma trivolvis*-infected C_3H/HeN mice, indicating that immunosuppression due to dexamethasone increased parasite infection (Fujino, et al., 1997). Ketotifen functions as an antagonist against histamine and a membrane stabilizer in eosinophils (Podleski, et al., 1984). In addition, ketotifen inhibits IL-5 and IgA secretion, and eosinophil activation (Dologalska, et al., 2000). Our results demonstrated that ketotifen inhibited worm recovery rate and shortened the infection period (Table 1 and Figs. 1~2). We cannot explain the exact immune mechanism in this ketotifen-induced eosinophil inhibition. A detailed mechanism is under investigation.

Parasites increase serum immunoglobulins in a host (Ito, et al., 1976; Kojima, et al., 1972) and eosinophil infiltration into a tissue (Ratmatunga, et al., 1999; Saito, et al., 1996). This study showed that ketotifen decreased IgA, IgE, and IgG in the *E. hortense*-infected mice (Figs. 4~7), possibly indicating that ketotifen is associated with eosinophil response.

IL-5 production and eosinophil infiltration is increased in *Angiostrongylus cantonensis*-infected mice (Korenaga, et al., 1994; Saski, et al., 1993). Miller et al. (1990) reported that eosinophil infiltration in *Hymenolepis diminuta* infection was affected by IL-5 and IgG1 production. These data indicate that Th2 cytokine induces eosinophil activation in parasite infection. Our results demonstrated that ketotifen decreased serum IL-4 and eosinophil infiltration, in agreement with previous reports.

In conclusion, we demonstrated that ketotifen inhibited the rapid expulsion of *E. hortense* and eosinophil infiltration in the intestinal mucosa of BALB/c mice. In addition, ketotifen decreased the concentration of serum IFN-γ and IL-4, indicating that ketotifen may play an important role in allergy response induced by *E. hortense*.

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