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The Alteration of Cytokine Expression and Goblet Cell Response by Cyclosporin A and Histamine Receptor Antagonists in C3H/HeN Mice Infected with *Echinostoma hortense*

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Echinostoma hortense (*E. hortense*) is an intestinal trematode with the highest infection rate in South Korea. However, the immune response against *E. hortense* infection has not been explained well. In the present study, we investigated the effect of treatment with cyclosporin A (CsA) and histamine receptor antagonists on the cytokine expression and mucosal goblet cells in *E. hortense*-infected C3H/HeN mice. The alteration of cytokine mRNA expression (TNF-α, IL-1β, IL-4 and IL-5), intestinal worm recovery rate and goblet cell responses were measured weekly from 0 to 5 weeks post-infection (P.I.) in the control and the following three drug-treated groups: CsA, hydroxyzine and cimetidine. Compared with the control group, the expression of TNF-α, IL-4 and IL-5 mRNAs decreased in the CsA- and hydroxyzine-treated groups, but only IL-4 mRNA expression did in the cimetidine-treated group. Worm recovery rate was significantly increased in the drug-treated groups. Mucosal goblet cells and their mucin response significantly decreased in the CsA-treated group ($P<0.01$), but significantly increased in the cimetidine- ($P<0.05$) and hydroxyzine- ($P<0.01$) treated groups. These data suggest that CsA treatment inhibits production of Th1- and Th2-type cytokines which are necessary for the worm expulsion. Histamine receptor increases goblet cells and their mucin activation, although it remains to be elucidated whether it directly affects the worm expulsion period of *E. hortense* in C3H/HeN mice.

Key Words: *Echinostoma hortense*, Cyclosporin A, Hydroxyzine, Cimetidine, Goblet cells, TNF-α, IL-4, IL-5

INTRODUCTION

Echinostoma hortense (*E. hortense*) was firstly found in 1926 (Asada, 1926) and the first case of human infection in South Korea was reported in 1983 (Seo et al., 1983). Many studies have reported about parasite expulsion, specifically the nematoda including *Trichinella spiralis*, *Nippostrongylus brasiliensis*, and *Strongyloides ratti* and the trematoda including *Metagonimus yokogawai*, and *Neodiplostomum seoulense* (Chai et al., 1993; Wakelin et al., 1993). Since an infecting parasite is excreted by the host immune response, a continuous pathogenesis is not shown in the parasite-infected host. The mechanism of parasite excretion is associated with T cell-dependent and -independent mechanisms.

Goblet cells exist in the columnar or pseudostratified columnar cells of intestine mucosa and they increase the production of mucin and glycoprotein. These substances play a protective role in the host against invading microorganisms (Meslin et al., 1999). The hypertrophy of goblet cells and the increased mucin secretion are associated with parasite expulsion, and specifically with that of the nema-

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Table 1. Primers used for PCR

Cytokine		Primer sequence (5' → 3')	Expected amplified fragment size (bp)
β-actin	Sense	AGG CTG TGC TGT CCC TGT ATC C	395
	Antisense	ACC CAA GAA GGA AGG CTG GAA A	
TNF-α	Sense	ATG AGC ACA GAA AGC ATC CGC	692
	Antisense	CCA AAG TAG ACC TGC CCG GAC TC	
IL-1β	Sense	GCT ACC TGT GTC TTT CCC GTG G	291
	Antisense	TTG TCG TTG CTT GGT TCT CCT TG	
IL-4	Sense	TCT CTA GAT CAT GGG CAT TTT GAA CGA GGT C	306
	Antisense	TGC ATG ATG CTC TTT AGG CTT TCC	
IL-5	Sense	ATG ACT GTG CCT CTG TGC CTG GAG C	243
	Antisense	CTG TTT TTC CTG GAG TAA ACT GGG G	

tode family including *Schistosoma mansoni*, *Trichinella spiralis*, *Nippostrongylus brasiliensis*, *Strongyloides ratti*, *Trichuris muris*, and *Echinostoma trivolvis* and trematoda (Miller and Nawa, 1979; Carroll et al., 1984; Miller, 1987; Grencis et al., 1991; Garside et al., 1992; Fujino et al., 1993; Khan et al., 1995; Ishikawa et al., 1997; Fallon et al., 2000; Khan et al., 2001; Khan et al., 2003). It has been reported that the alteration of the mucin terminal sugar during worm expulsion is a more essential factor than the elevated number of goblet cells (Ishikawa et al., 1993; Ishikawa, 1994; Onah and Nawa, 2000).

Cyclosporin A (CsA) is well known as an immune suppressive agent. The CsA-induced mechanism includes the inhibition of T cell reaction and mast cell proliferation (Ryffell, 1993). The alteration of smooth muscle cells and mucin glands has been reported in animal models after CsA treatment, along with the hypertrophy of goblet cells (Padrid et al., 1996). Histamine plays an important role in a pathophysiological process of allergic inflammation (Jutel et al., 2005). Both histamine H1 receptor (H_1R) and H2 receptor (H_2R) antagonists have therapeutic effects in allergic dermatitis (Simons, 1995).

In the current study, we examined the worm recovery rate and the alteration of cytokine expression, goblet cell numbers and mucin secretion in *E. hortense*-infected C3H/HeN mice after CsA, hydroxyzine (H_1R antagonist) or cimetidine (H_2R antagonist) treatment.

MATERIALS AND METHODS

1. Experimental animals

Five-week-old C3H/HeN mice were obtained from the Orientbio Animal Center. The experimental groups of three mice each were divided into a control group and three drug-treated groups. *E. hortense* metacercariae were collected by an artificial digestion of *Misgurnus anguillicaudatus* and thirty metacercariae were fed to each mouse via a stomach tube. The daily administrations of CsA, cimetidine and hydroxyzine treatment in the drug-treated groups were 7.5 mg/kg), 20 mg/kg, and 1 mg/kg, respectively.

2. Worm recovery rate

The mice infected with metacercariae were killed at one week intervals over 5 weeks. For worm recovery, the small intestine was excised, placed in 1M PBS, and incubated for 2 h, after which the worms were collected.

3. Semiquantiative RT-PCR

The splenocyte cells were isolated from the experimental mice and centrifuged at 1,500 g for 5 min. After removal of the supernatants, the total RNA was extracted with Trizol reagent (Life Technologies, NY), as described in the user's manual. Reverse transcription was constituted by using AccuPower RT PreMix (Bioneer, Seoul, Korea) with 200 pmole of oligo dT18. The extension reaction was carried out at 42℃ for 60 min. The reaction was terminated by incubations at 95℃ for 5 min. The cDNA products were subsequently amplified with 0.025 U of TaKaRa TaqTM (Takara, Japan) in a 50 μl reaction mixture containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 1 μM of the forward and reverse primers. The primer sequences and the product lengths are described in Table 1. After denaturation at 94℃ for 5 min, each cytokine underwent the following thermocycling: 94℃ for 30 sec, 60℃ for 30 sec and 72℃ for 1 min for the IL-4, IL-5, IL-1β and β-actin; 94℃ for 30 sec, 65℃ for 30 sec and 72℃ for 40 sec for TNF-α (Ed- how

many cycles were operated? Your description implies only one cycle, 2 minutes for the former and 1min40sec for the latter, but I presume there were several such cycles). β-Actin was used as an internal control for each PCR reaction. The PCR products were analyzed via 1% agarose gel electrophoresis, followed by ethidium bromide staining.

4. Periodic acid Schiff (PAS) staining for goblet cell detection

The upper part of the small intestine was divided into 3 cm sections, washed with saline and fixed with Carnoy's solution. The tissues were embedded in paraffin, cut with a microtome and stained with PAS. Hematoxylin was used as the counterstain. The number of goblet cells was analyzed according to a previously described method (Miller et al., 1987). Ten villi were counted in each region of the intestine and all counts were shown as the number of cells per villus-crypt unit (VCU).

5. Lectin staining for mucin response of goblet cells

Lectin immunohistochemistry was carried out using helix pomatia agglutinin (HPA; Sigma, St. Louis, MO), as described by the manufacturer's instruction, for examining the goblet cell activation. HPA binds to the modified N-Acetyl-D-galactosamine on GC mucin. The tissue sections were fixed with Carnoy's solution, embedded with paraffin and then sectioned to 4 μm thickness. The tissues were deparaffinized, hydrolyzed with 100%, 90%, 80% and 70% ethanol, and incubated for 20 min with 0.3% H_2O_2 in methanol for the removal of the peroxidase activity. After washing the sections 3 times with 0.01 M PBS (pH 7.4), the non-specific antibody binding was diminished by incubating the tissues in 1% bovine serum albumin. The tissue sections were incubated at a biotinylated HPA concentration of 25 μg/ml in a moist chamber for 2 hours, and then washed with PBS solution and incubated with biotin-conjugated, rabbit anti-goat IgG (DAKO, Glostrup, Denmark). After incubation with streptavidin-HRP conjugate (Zymed, San Francisco, U.S.A) at a 1:200 dilution for 2 h, the tissues were developed. Mayer's hematoxylin was used as the counterstain. All counts were presented as the number of HPA-positive cells per 10 VCU.

6. Statistical analysis

The data are presented as means ± SD. Statistical differences were analyzed by using the paired t test for determining the difference between the control and drug-treated groups. The SPSS statistical software package (Version 10.0, Chicago, IL) was used for statistical analysis. P value < 0.05 was considered statistically significant.

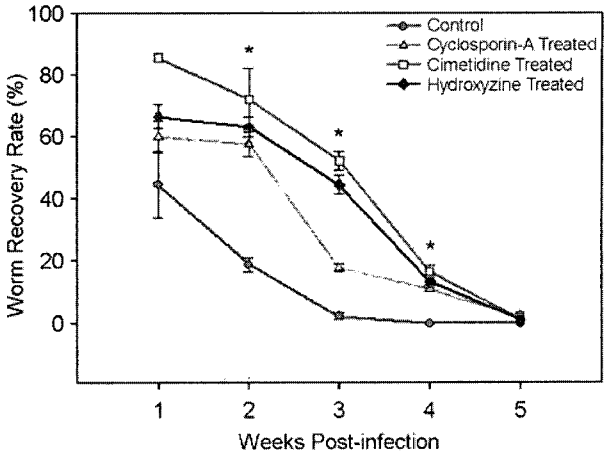

Fig. 1. Worm recovery rate of *E. hortense* in the control and drug-treated C3H/HeN mice infected with *E. hortense*. Thirty *E. hortense* metacercariae were orally introduced into the C3H/HeN mice. The infected mice were treated in the absence (●) or presence of cyclosporin A (▲), hydroxyzine (◆) or cimetidine (■). One mouse was sacrificed every week, and the adult worms parasitizing in the intestines were counted. The data are expressed as the mean ratio of the number of collected worms to the number of introduced worms, and were represented as the mean ± SD of three independent experiments. *P<0.05 was used to determine statistically significant difference between the control and drug-treated groups.

RESULTS

1. Worm recovery rate of the C3H/HeN mice

The worms were collected from the mice infected with metacercariae at one-week intervals for 5 weeks. The number of worms recovered in the control group was 44.4± 18.4, 18.9±3.8 and 2.2±1.9 at 1, 2 and 3 weeks post-infection (P.I.), respectively. No worms were recovered after week 4 P.I. (Fig. 1). The rate in the drug-treated groups was significantly decreased from week 2 P.I. to week 4 P.I in comparison with the control group (P<0.05) (Fig. 1). These results show that the drugs delayed the worm excretion period.

2. Cytokine mRNA expression in the splenocytes of the C3H/HeN mice

We next examined cytokine mRNA level by performing

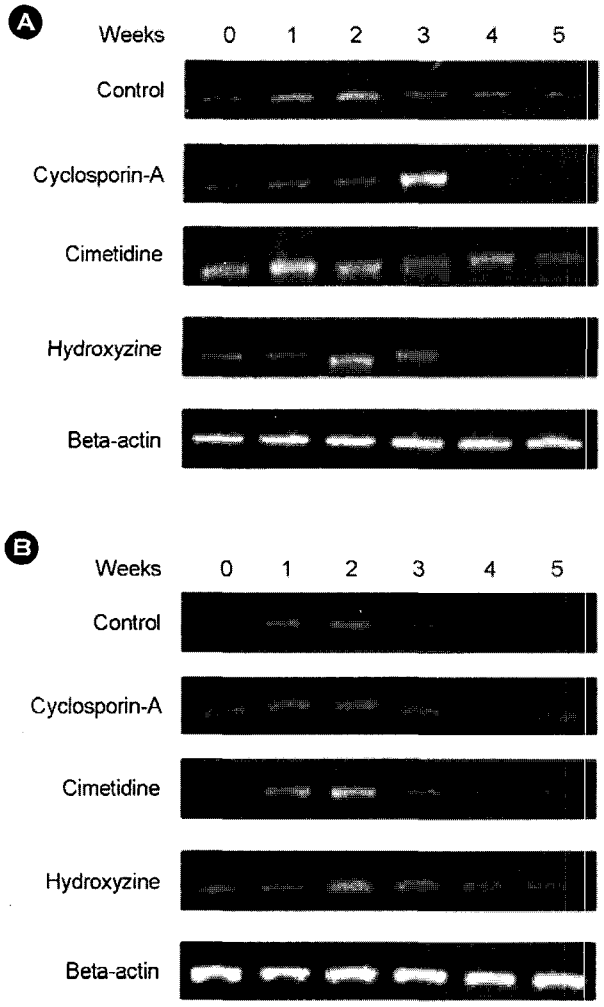

Fig. 2. Expression of TNF-α and IL-1β mRNAs in the control and drug-treated C3H/HeN mice infected with *E. hortense*. RNA was extracted from splenocytes from the C3H/HeN mice. RT-PCR was performed for TNF-α **(A)** and IL-1β **(B)** mRNAs by using the templates isolated from the cells. β-Actin was used as an internal control. The products were visualized by 1% agarose gel electrophoresis.

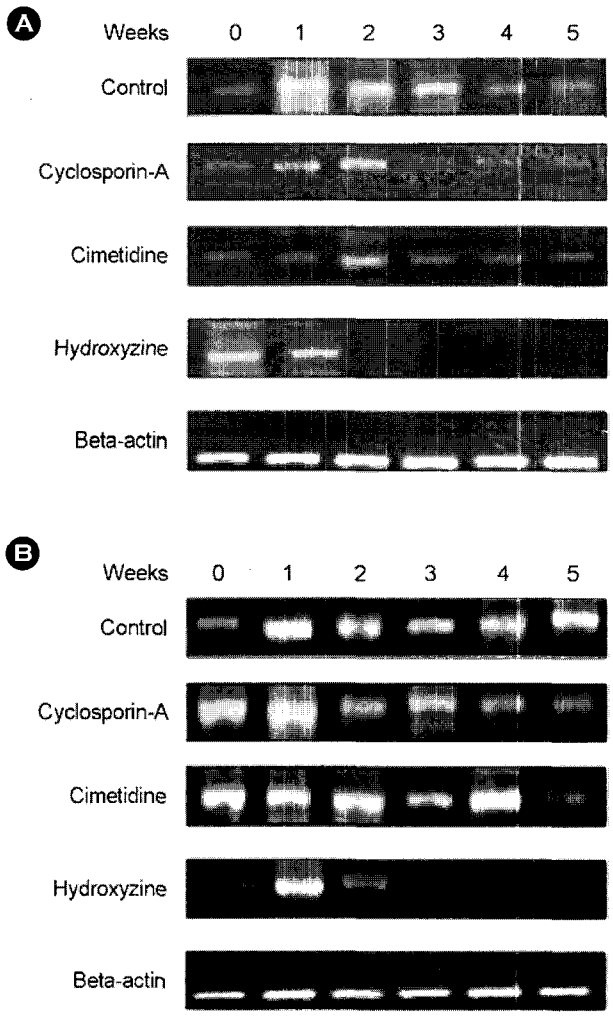

Fig. 3. Expression of IL-4 and IL-5 mRNAs in the control and drug-treated C3H/HeN mice infected with *E. hortense*. RNA was extracted from splenocytes from the C3H/HeN mice. RT-PCR was performed for IL-4 **(A)** and IL-5 **(B)** mRNAs by using the templates isolated from the cells. β-Actin was used as an internal control. The products were visualized by 1% agarose gel electrophoresis.

RT-PCR for TNF-α, IL-1β, IL-4, and IL-5. Fig. 2 shows the alternation of TNF-α and IL-1β mRNA expression in the splenocytes after *E. hortense* infection. Both mRNA expressions were increased in the splenocytes at week 1 P.I., peaked at week 2 P.I. and then decreased until week 5 P.I. Both CsA and hydroxyzine treatment completely inhibited the expression of TNF-α mRNA at weeks 4 and 5 P.I. (Fig. 2A). However, IL-1β mRNA expression was not affected by drug treatment (Fig. 2B). The expression of IL-4 and IL-5 mRNA increased at week 1 P.I. and, although slightly decreased, lasted until week 5 P.I. (Fig. 3). Both CsA and hydroxyzine, but not cimetidine, treatment blocked IL-4 and IL-5 mRNA expression, indicating that CsA and hydroxyzine block the expression of Th1- and Th2-type cytokines.

3. Goblet cell response in the intestinal mucosa of the C3H/HeN mice

As shown in Fig. 4, the number of goblet cells in the duodenum of the control group was 98.7±3.2 at baseline, increased rapidly at week 1, peaked at week 3 P.I. (175.3± 8.4), and then declined gradually until week 5 P.I. In the duodenum of the CsA-treated group, the number was 109.7 ±11.8 at baseline, continued to increase until a peak at

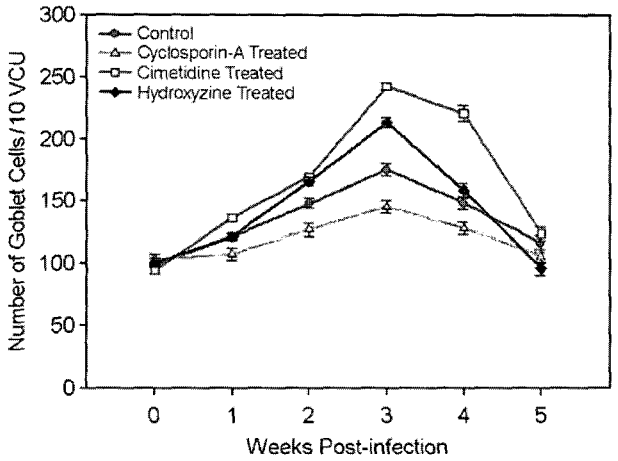

Fig. 4. Number of mucosal goblet cells per 10 villus-crypt unit (VCU) in the control and drug-treated C3H/HeN mice infected with *E. hortense*. The infected mice were treated in the absence (●) or presence of cyclosporin A (▲), hydroxyzine (◆) or cimetidine (■). As described in the Methods section, a tissue section of intestine was stained by PAS and counted with a microscope. The data were represented as the mean ± SD of three independent experiments.

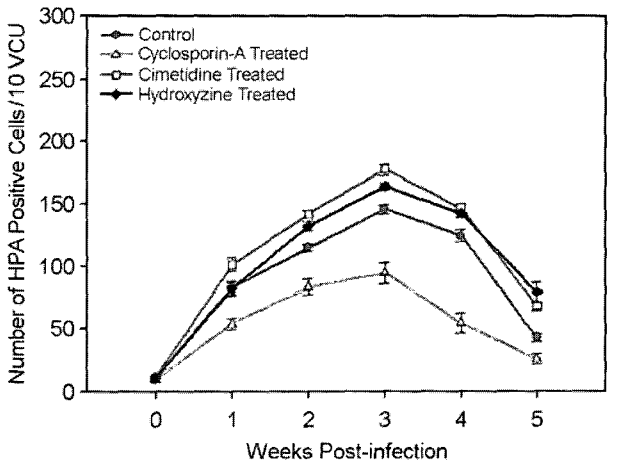

Fig. 5. Number of HPA (helix pomatia agglutinin) positive cells per 10 villus-crypt unit (VCU) in the small intestine of C3H/HeN mice infected with *E. hortense*. The infected mice were treated in the absence (●) or presence of cyclosporin A (▲), cimetidine (■), or hydroxyzine (◆). As described in the Methods section, a tissue section of intestine was stained by HPA and counted with a microscope. The data were represented as the mean ± SD of three independent experiments.

week 3 P.I. (145.3±9.3), and then declined. The number of goblet cells in the CsA-treated group was decreased compared to the control group throughout the experimental period, whereas in the cimetidine- and hydroxyzine-treated groups it was significantly increased at week 3 P.I. to 242.7 ±5.7 and 213.3±6.7, respectively, in comparison with the control group (P<0.01). These results indicated that CsA treatment strongly inhibited the goblet cell response in the C3H/HeN mice, whereas cimetidine and hydroxyzine treatment activated the response.

4. Mucin response of goblet cells using lectin histochemistry

Because the number of goblet cells was altered by CsA, cimetidine and hydroxyzine treatment, we examined whether the mucin response of goblet cells is affected by the drug treatment (Fig. 5). In the duodenum of the control group of the C3H/HeN mice, the number of HPA-positive cells was was increased at week 1 to a peak at week 3 P.I. (145.7±5.7), and then declined until week 5 P.I. (42.3±5.7). Although mucin response in the drug-treated groups showed a similar trend to the control group, the number of HPA-positive cells in the CsA-treated group was decreased compared to the control group, whereas that in the cimetidine- and hydroxyzine-treated groups was more elevated than in the control group. These results were consistent with the data from the number of goblet cells shown in Fig. 4. The highest number of HPA-positive cells was shown at week 3 P.I., when worm recovery rate rapidly lessened, which indicated that goblet cells play an essential role in immune defense against *E. hortense*.

DISCUSSION

In the present study, we examined the effects of CsA, cimetidine, and hydroxyzine treatment on C3H/HeN mice infected with *E. hortense* metacercariae. We demonstrated that, (1) CsA, cimetidine, and hydroxyzine treatment delayed the period of worm recovery rates in the C3H/HeN mice, (2) CsA and hydroxyzine treatment decreased TNF-α, IL-4, and IL-5 mRNA expression in the C3H/HeN mice, (3) cimetidine treatment inhibited only IL-4 mRNA level in the C3H/HeN mice, and (4) CsA treatment inhibited the number of goblet cells and mucin response in the C3H/HeN mice, whereas hydroxyzine and cimetidine increased them.

The mRNA expressions of Th1- and Th2-type cytokines, TNF-α, IL-1β, IL-4 and IL-5 were increased at week 1 or 2 P.I. with a peak level in the *E. hortense*-infected C3H/HeN mice (Figs. 2 & 3). Goblet cells peaked at week 3 P.I., when worm recovery rates declined (Figs. 1 & 4). Lectin histochemical analysis revealed increased mucin production in

the *E. hortense*-infected group, consistent with the increased number of goblet cells (Fig. 5). These results indicate the possibility that increased cytokine level activates goblet cell response which then leads the cells to increase their worm repulsion.

CsA is well known as an immunosuppressive agent with a variable effect depending on the type of parasite infection. Malaria, schistosome, adult tape worm, metacestode and filarial nematode were removed in the host by CsA treatment. However, CsA induced immunosuppression of the host in toxoplasma, avian coccidiosis and gastrointestinal nematode infection (Chappell and Wastling, 1992). In this study, the expressions of TNF-α, IL-4, and IL-5 mRNA in *E. hortense*-infected mice were inhibited by CsA treatment (Figs. 2 & 3). In addition, goblet cell response, including increased cell number and mucin secretion, was significantly blocked by CsA treatment (Figs. 4 & 5). These data indicate that CsA in *E. hortense* infection decreases the host immune response, including worm excretion by inhibiting cytokine expression and function of goblet cells.

Histamine plays an essential role in worm expulsion of the host by a variety of mechanisms, including contraction of intestinal smooth muscle and production of intracellular cyclic adenosine monophosphate (cAMP) (Mycek et al., 2000; Alewijnse et al., 1998). It has been reported that desloratadine inhibits IL-4 and IL-13 in basophils and that mepyramine blocks worm repulsion in guinea pigs infected with *Trichostrongylus colubriformis* (Schroeder et al., 2001; Rothwell et al., 1978). A recent study has reported that hydroxyzine and cimetidine inhibit the function of goblet cells in rats infected with *N. seoulense* (Shin et al., 2003). In investigating the effect of hydroxyzine and cimetidine on *E. hortense* infection, we found that both delayed the period of worm excretion during *E. hortense* infection. However, hydroxyzine, in contrast to cimetidine, inhibited the expression of TNF-α, IL-4, and IL-5 mRNAs. As shown in the unexpected results of Figs. 4 and 5, hydroxyzine increased the number of goblet cells and mucin-secreted cells. Future work is planned to confirm the precise mechanism of the histamine antagonist in the regulation of worm expulsion and goblet cell number during *E. hortense* infection and also to elucidate the function of CsA, cimetidine, and hydroxyzine in the host response during other parasite infections. In summary, we demonstrated that CsA inhibited worm expulsion by down-regulating the expression of Th1- and Th2-type cytokines, the number of goblet cells and mucin secretion. Although both hydroxyzine and cimetidine elevated the number of lectin-binding goblet cells in the intestinal mucosa, they blocked worm expulsion. This study may provide a clue to elucidate the mechanism of immune response in mice with a parasite infection.

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