J. Exp. Biomed. Sci. 2012, 18(3): 254~259
pISSN : 1738-3226

Original Article

Differential Cytokine and Immunoglobulin Expressions in the Small Intestine of *Echinostoma hortense* Infected BALB/c Mice

Yoon Kyung Jo[1], Dongsup Lee[2], Sung In Kim[3], Ji-Sook Lee[4], Ji-Eun Oh[5] and Ho Joong Sung[6,†]

[1]Department of Clinical Laboratory Science (CLS), Dongnam Health College, Soowon-si, Gyeonggyi-do 440-714, Korea
[2]Department of Biomedical Laboratory Science (BLS), Hyejeon College, Hongseong-goon, Choongchungnam-do 350-702, Korea
[3]BLS, College of Natural Science, Gimcheon University, Gimcheon-si, Gyungsangbook-do 740-704, Korea
[4]CLS, Wonkwang Health Science University, Iksan-si, Jeonrabook-do 570-750, Korea
[5]BLS, School of Public Health, Far East University, Eumseong-goon, Choongchungbook-do 369-700, Korea
[6]BLS, College of Health Science, Eulji University, Seongnam-si, Gyeonggyi-do 461-713, Korea

Infections involving *Echinostoma hortense* (*E. hortense*) are considered to more severe than infections caused by other heterophyids. Although parasite expulsion by host immune responses attenuates the symptoms of infection, the detailed mechanisms of the host immune response need to be determined, especially in local immune responses involving cytokine and immunoglobulin expressions. We infected BALB/c mice with *E. hortense* and examined recovery rates together with expressions of multiple cytokines and immunoglobulins in the villi and crypts of the small intestine using immunohistochemistry. We observed a close correlation between worm expulsion rates and cytokine/immunoglobulin expressions in *E. hortense* infected mice. This study contributes to an understanding of the relationship between the immune response and parasite expulsion in hosts.

Key Words: *Echinostoma hortense*, Cytokines, Immunoglobulins, Small intestines, Immunohistochemistry

INTRODUCTION

E. hortense belongs to the class of intestinal trematoda that is associated with human infection (Cho et al., 2003). *E. hortense* has been found to infect humans, especially in East Asia, including Japan, China, and South Korea (Ahn and Ryang 1986). In South Korea, the first case of human infection was reported in 1983 (Seo et al., 1983). The symptoms of *E. hortense* infection are abdominal pain, diarrhea, and easy fatigability (Chai and Lee 1990). Infections with *E. hortense* eventually become more severe than infections with other heterophyid species (Chai et al., 2005; Youssef et al., 1987). Previous study has shown that *E. hortense* causes severe ulceration and mucosal damage (Chai et al., 1994; Lee et al., 2004). Although intestinal flukes cause human infection, parasites can be excreted by host immune responses, and pathogeneses are attenuated in infected hosts (Kim et al., 2000). The mechanism of parasite excretion is correlated with T cell-dependent or independent mechanisms. T cells stimulate the production of cytokines, including Interleukin-4 (IL-4) and IL-5, and regulate the production of antibodies against parasites (Ryang et al., 2007). Increased cytokine levels augment the growth and accumulation of eosinophils, mucosal mast cells, and goblet cells (Saito et al., 1996; Togawa et al., 2001; Urban et al., 1991). Cytokine stimulating mast cells increase production of IL-1 and tumor necrosis factor-alpha

*Received: August 1, 2012 / Revised: August 20, 2012
Accepted: August 20, 2012
†Corresponding author: Ho Joong Sung. Department of Biomedical Laboratory Science College of Health Science Eulji University 212 Yangji-dong, Soojeong-gu, Sungnam-si, Gyeonggi-do 461-713, Korea.
Tel: +82-31-740-7306, Fax: +82-31-740-7354
e-mail: hjsung@eulji.ac.kr

(TNF-α), and goblet cells enhance mucin production, all of which induce expulsion of parasites (Miller 1996; Ryang et al., 2007; Shakoory et al., 2004). The anti-allergy drug ketotifen consistently decreases expression of IL-4, serum T-helper (Th) type 2 cytokines, and Immunoglobulin A (IgA), and regulates parasite recovery rates in *E. hortense* infected mice (Ryang et al., 2007). Based on the important function of cytokines in parasite infected hosts, multiple studies have focused on the regulatory mechanism of cytokines in either a T cell-dependent or independent manner. However, as intestinal mucosal cells are known to be a regulator of cytokine expression in parasite infected hosts, cytokine expression in intestinal mucosal cells has also been studied. Herein, we examined the expression levels of multiple cytokines (IgA and E) in the small intestine of *E. hortense* infected BALB/c mice using immunohistochemistry.

MATERIALS AND METHODS

Materials

All antibodies used in immunohistochemical analysis were purchased from R&D systems Inc. (R&D systems Inc, Minneapolis, MN, USA). All chemicals used in experiments were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). BALB/c mice were provided by Central Lab. Animal Inc. (Central Lab. Animal Inc, Seoul, Korea). A Peroxidase/DAB kit was purchased from Dako (Dako, Carpinteria, CA, USA). Mounting solution with FITC was provided by Vector Labs. (Vector Labs., Burlingame, CA, USA).

Animal experiments

Six-week old male mice were orally infected with 50 metacercariae of *E. hortense*. Infected mice were sacrificed at days 5, 25, and 45. All animal experiments were conducted under National Institutes of Health (NIH) animal care guidelines.

Worm recovery rate

To examine the worm recovery rate, the small intestine was incubated in a pre-warmed 1 M PBS solution for 2 h, followed by collection of adult *E. hortense* specimens. The recovery rate was calculated as: Recovery rate (%) = the number of recovered adult *E. hortense* specimens/the number of infecting metacercariae × 100.

Table 1. The worm recovery rate in *E. hortense*

Infection period (day)	Recovery rate (%)
5	55.3 ± 7.5
25	10.5 ± 6.7
45	0.3 ± 0.7

*Data are shown as mean ± S.D.
*Six BALB/c mice were orally infected with 50 metacercariae in each group.

Immunohistochemistry

Detailed methods previously described were used (Ryang et al., 2003). Briefly, frozen tissue sections (5 μm) were fixed in cold acetone for 10 min. To examine the expression levels of IL-4, IL-5, TNF-α, and Interferon-gamma (INF-γ), streptavidin-biotin methods were used following the manufacturer's instructions. Vectashield mounting medium with FITC was used to detect both IgA and E. Slides were observed under a fluorescence microscope (Olympus, Tokyo, Japan) and images were captured using a single-lens reflex camera (Nikon, Tokyo, Japan). To determine the expression levels of cytokines and immunoglobulins in the small intestine, cytokine and immunoglobulin producing cells were counted in the crypt and villi from 10 randomly selected lesions.

Statistical analysis

P values were calculated using Student's *t*-test as indicated.

RESULTS

Worm recovery rates in *E. hortense* infected BALB/c mice

Mice were orally infected with 50 metacercariae and six infected mice were sacrificed at days 5, 25, and 45 (Table 1). We observed 27.7 adult *E. hortense* specimens (a 55.3% recovery rate) in the small intestine at day 5. The recovery

rate decreased to 10.5% at day 25, and almost all adult *E. hortense* specimens were discharged by day 45 (a 0.3% recovery rate).

Various cytokine expressions in the small intestine of *E. hortens* infected mice

Multiple studies have reported that IL-4, IL-5, TNF-α, and INF-γ are correlated with parasite expulsion (Kopf et al., 1995; Winsor et al., 2000; Yu and Perdue 2001). Therefore, we examined expression levels of these cytokines in the dissected small intestine of *E. hortense* infected mice using an immunohistochemical method. The expression levels of cytokines were determined by counting the number of cells that strongly produced cytokines under microscopic examination (Fig. 1A, Fig. 2). The number of cells that produced the cytokines IL-4, IL-5, TNF-α, and INF-γ was less than 2 in both the villi and crypts of an uninfected mice control (Fig. 1A). The number of cytokine producing cells was significantly increased ($P < 0.05$) at infection day 25 in villi, and either continuously increased or maintained up to infection day 45 in villi. The number of IL-4 producing cells in crypts was significantly increased at infection day 5, and then the cell number gradually decreased up to day 45 (Fig. 1A). For IL-5 and INF-γ producing cells, the cell

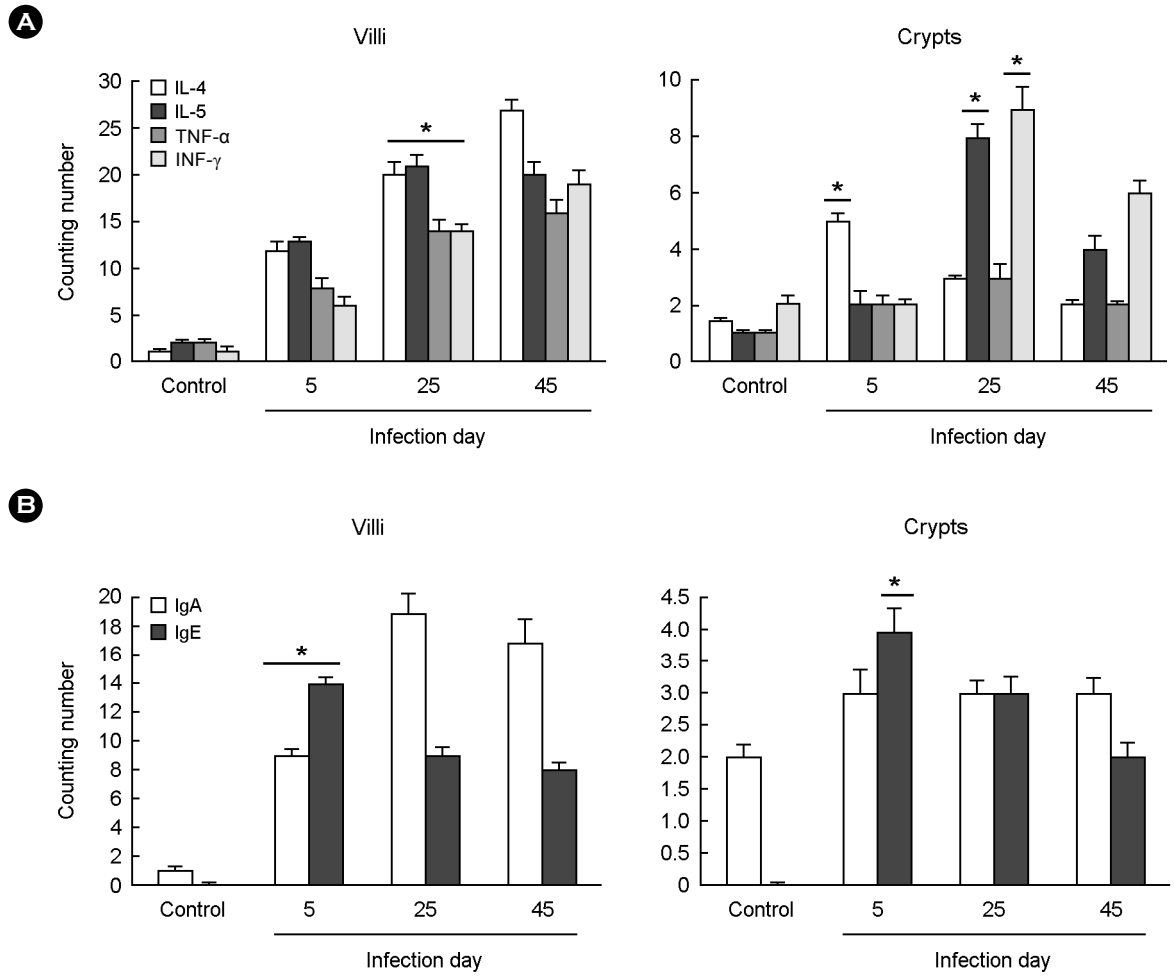

Fig. 1. Analysis of various cytokine expressions in the small intestine. Fifty metacercariae infected mice were sacrificed at days 5, 25, and 45, together with uninfected mice. Small intestines were subsequently dissected. Frozen sections were stained with IL-4, IL-5, TNF-α, and IFN-γ antibodies, and slides were examined under a light microscope (magnification ×40) (A). Cytokine expressing cells were counted in randomly selected villi (left panel) and crypts (right panel). Data are shown as mean ± S.D. (*: $P < 0.05$). Using IgA and IgE antibodies, the expression levels of immunoglobulins were observed under a fluorescence microscope (magnification ×40) (B). Methods of cell counting and statistical analysis were the same as for Fig. 1A.

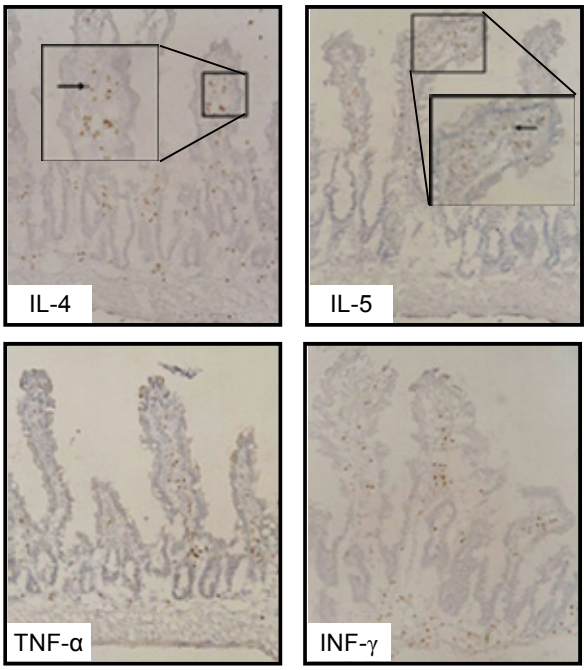

Fig. 2. Representative images of cytokine expressions. All presented images were obtained at infection day 25 (magnification ×40). Smaller images showing IL-4 and IL-5 stained results were enlarged to larger images, as shown. Arrows indicate representative IL-4 and IL-5 expressions in each image.

number was significantly increased at infection day 25 in crypts and decreased by day 45. However, the TNF-α producing cell number did not significantly change during the infection periods.

IgA and IgE expressions in the small intestine of *E. hortense* infected mice

Previous reports have shown that the expression levels of IgA and IgE are affected by parasite infections (Akao et al., 1990; Bueno et al., 2000). Together with the examination of various cytokine expressions in both the villi and crypts of *E. hortense* infected mice, we also observed the expression levels of IgA and IgE using fluorescence microscopic examination (Fig. 1B and Fig. 3). The method to determine the expression level of immunoglobulins was the same as for cytokines. The number of IgA producing cells was less than 2 in both the villi and crypts of uninfected control mice. The number of IgA producing cells was significantly increased ($P < 0.05$) at infection day 5 and increased even more by day 45 in the villi. However,

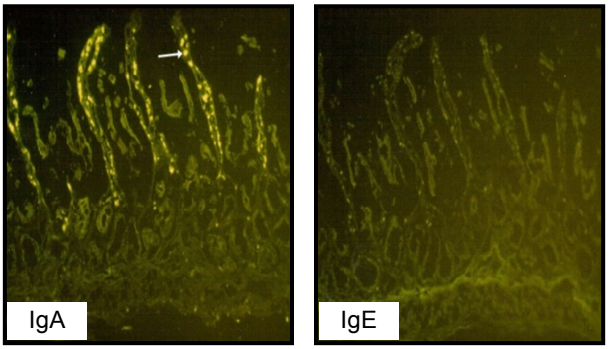

Fig. 3. Representative images of immunoglobulin expressions. All presented images were obtained at infection day 25 (magnification ×40). The white arrow indicates a representative image of IgA expression.

the IgA producing cell number was not significantly increased in crypts during the infection periods. Statistical analysis showed that fewer than one cell produced IgE in the villi and crypts of control mice (Fig. 1B). However, the number of IgE producing cells was significantly increased at infection day 5 in both the villi and crypts, but was decreased during longer infection periods.

DISCUSSION

Examination of host immune responses using parasite infected local lesions is essential to study parasite expulsion (Ishikawa et al., 1993; Miller and Jarrett 1971). Therefore, assessment of cytokine and immnunoglobulin expression levels in the small intestine of *E. hortense* infected mice using immunohistochemistry can be more valuable than examinations using other methods. In this study, we infected BALB/c mice with the metacercariae of *E. hortense* and observed worm recovery rates in a time-dependent manner. We also dissected the small intestine from *E. hortense* infected mice and observed the expression levels of multiple cytokines and Immunoglobulins. As shown Fig. 1A, expression levels of the cytokines IL-4, IL-5, TNF-α, and INF-γ were continuously increased in villi up to infection day 25 for IL-5, and 45 for IL-4, TNF-α, and INF-γ. IL-4 expression in crypts was decreased after infection day 5 during longer infection periods. The expression of IL-5, and INF-γ was decreased after day 25. Interestingly, TNF-α expression was not significantly increased or decreased in

crypts. Comparing the expression pattern of cytokines between villi and crypts, the expression level of all cytokines, except TNF-α, was attenuated after day 5 (IL-4) and day 25 (IL-5, and INF-γ) in crypts. However, the expression levels of all observed cytokines were increased in villi. These results are probably associated with the characterization of local immune responses in the small intestine. A previous report showed that immune responses were initiated in crypts early in infection, and that responses were more activated in villi (Owen and Jones 1974). Concerning the correlation between worm recovery rates and cytokine expression, 89.5% of adult *E. hortense* specimens were discharged by infection day 25 (Table 1), which is the time point where expression levels of all cytokines were significantly increased in the villi (Fig. 1A). The expression of IgE was significantly increased at infection day 5 in both villi and crypts. IgA expression was only significantly increased in villi (infection day 5) and not in crypts (Fig. 1B). Herein, we examined cytokine expression levels in both the villi and crypts of *E. hortense* infected mice using direct observation. Although we did not identify which immune cells responded to parasite infection and subsequently expressed cytokines and immunoglobulins in the small intestine, we did observe cytokine/immunoglobulin expressions directly in a time-dependant manner in a parasite infected host. In addition, we have shown that expressions of IL-4, IL-5, TNF-α, and INF-γ in the villi are implicated in parasite expulsion, based on worm recovery rates. Further study is needed to determine the molecular mechanism of immune responses in the small intestine of *E. hortense* infected hosts, and to define other cytokines that regulate parasite expulsion. This study contributes to an understanding of the relationship between the immune response and parasite expulsion in an infected local lesion.

Acknowledgements

We are grateful to Prof. Yong Suk Ryang for helpful suggestions and support. This work was supported by the Research Fund (2011) from the Dongnam Health College.

REFERENCES

Ahn YK, Ryang YS. [Experimental and epidemiological studies on the life cycle of *Echinostoma hortense* Asada, 1926 (Trematoda: Echinostomatidae)]. Kisaengchunghak Chapchi. 1986. 24: 121-136.

Akao N, Ohyama T, Kondo K. Immunoblot analysis of serum IgG, IgA and IgE responses against larval excretory-secretory antigens of Anisakis simplex in patients with gastric anisakiasis. J Helminthol. 1990. 64: 310-318.

Bueno EC, Vaz AJ, Machado LD, Livramento JA. Neurocysticercosis: detection of IgG, IgA and IgE antibodies in cerebrospinal fluid, serum and saliva samples by ELISA with Taenia solium and Taenia crassiceps antigens. Arq Neuropsiquiatr. 2000. 58: 18-24.

Chai JY, Darwin Murrell K, Lymbery AJ. Fish-borne parasitic zoonoses: status and issues. Int J Parasitol. 2005. 35: 1233-1254.

Chai JY, Hong ST, Lee SH, Lee GC, Min YI. A case of echinostomiasis with ulcerative lesions in the duodenum. Korean J Parasitol. 1994. 32: 201-204.

Chai JY, Lee SH. Intestinal trematodes of humans in Korea: Metagonimus, heterophyids and echinostomes. Kisaengchunghak Chapchi. 1990. 28: 103-122.

Cho CM, Tak WY, Kweon YO, Kim SK, Choi YH, Kong HH, Chung DI. A human case of *Echinostoma hortense* (Trematoda: Echinostomatidae) infection diagnosed by gastroduodenal endoscopy in Korea. Korean J Parasitol. 2003. 41: 117-120.

Ishikawa N, Horii Y, Nawa Y. Immune-mediated alteration of the terminal sugars of goblet cell mucins in the small intestine of Nippostrongylus brasiliensis-infected rats. Immunology. 1993. 78: 303-307.

Kim I, Im JA, Lee KJ, Ryang YS. Mucosal mast cell responses in the small intestine ats infected with *Echinostoma hortense*. Korean J Parasitol. 2000. 38: 139-143.

Kopf M, Le Gros G, Coyle AJ, Kosco-Vilbois M, Brombacher F. Immune responses of IL-4, IL-5, IL-6 deficient mice. Immunol Rev. 1995. 148: 45-69.

Lee KJ, Park SK, Im JA, Kim SK, Kim GH, Kim GY, Yang EJ, Ryang YS. Susceptibility of several strains of mice to *Echinostoma hortense* infection. Korean J Parasitol. 2004. 42: 51-56.

Miller HR. Mucosal mast cells and the allergic response against nematode parasites. Vet Immunol Immunopathol. 1996. 54: 331-336.

Miller HR, Jarrett WF. Immune reactions in mucous membranes. I. Intestinal mast cell response during helminth expulsion in the rat. Immunology. 1971. 20: 277-288.

Owen RL, Jones AL. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. Gastroenterology. 1974. 66: 189-203.

Ryang YS, Im JA, Kim IS, Kim KH. Mucosal Mast Cell Responses in the Small Intestine of C3H/HeN and BALB/c Mice Infected with *Echinostoma hortense*. J Biomed Lab Sci. 2003. 9: 145-150.

Ryang YS, Yang EJ, Kim JL, Lee KJ, Sung HJ, Kim JB, Kim IS. Immune response and inhibitory effect of ketotifen on the BALB/c and C3H/HeN mice infected with *Echinostoma hortense*. Parasitol Res. 2007. 101: 1103-1110.

Saito S, Hamada A, Watanabe N, Obata T, Katakura K, Ohtomo H. Eosinophil chemotactic activity in Leishmania amazonensis promastigotes. Parasitol Res. 1996. 82: 485-489.

Seo BS, Hong ST, Chai JY, Lee SH. Studies On Intestinal Trematodes In Korea: VIII. A Human Case Of *Echinostoma Hortense* Infection. Kisaengchunghak Chapchi. 1983. 21: 219-223.

Shakoory B, Fitzgerald SM, Lee SA, Chi DS, Krishnaswamy G. The role of human mast cell-derived cytokines in eosinophil biology. J Interferon Cytokine Res. 2004. 24: 271-281.

Togawa M, Kiniwa M, Nagai H. The roles of IL-4, IL-5 and mast cells in the accumulation of eosinophils during allergic cutaneous late phase reaction in mice. Life Sci. 2001. 69: 699-705.

Urban JF, Jr., Katona IM, Paul WE, Finkelman FD. Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. Proc Natl Acad Sci U S A. 1991. 88: 5513-5517.

Winsor GL, Waterhouse CC, MacLellan RL, Stadnyk AW. Interleukin-4 and IFN-gamma differentially stimulate macrophage chemoattractant protein-1 (MCP-1) and eotaxin production by intestinal epithelial cells. J Interferon Cytokine Res. 2000. 20: 299-308.

Youssef MM, Mansour NS, Awadalla HN, Hammouda NA, Khalifa R, Boulos LM. Heterophyid parasites of man from Idku, Maryut and Manzala lakes areas in Egypt. J Egypt Soc Parasitol. 1987. 17: 475-479.

Yu LC, Perdue MH. Role of mast cells in intestinal mucosal function: studies in models of hypersensitivity and stress. Immunol Rev. 2001. 179: 61-73.