Echinostoma hortense

Th2 cytokine
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1. mouse splenocytes
2. Echinostoma hortense
3. Echinostoma hortense
4. Total RNA
5. Reverse transcription polymerase chain reaction (RT-PCR)
6. ELISA

3. Echinostoma hortense
1. Echinostoma hortense
2. Lipopolysaccharide (LPS)
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Ab : antibody
BSA : bovine serum albumin
DEPC : diethyl pyrocarbonate
DMEM : Dulbecco's modified Eagle's minimum essential medium
DMSO : dimethyl sulfoxide
ELISA : enzyme-linked immunosorbent assay
FBS : fetal bovine serum
Ig : immunoglobulin
IL : interleukin
LPS : lipopolysaccharides
MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide
PBS : phosphate buffered saline
PBST : phosphate buffered saline-Tween 20
RBC : red blood cell
mRNA : messenger RNA (ribonucleic acid)
RT-PCR : reverse transcription polymerase chain reaction
TBE : Tris-borate/EDTA
Echinostoma hortense

Th2 cytokine

6 BALB/c mouse spleen primary cell culture splenocytes Echinostoma hortense crude extract antigen (excretory-secretory product; E.S.P.) lipopolysaccharide (LPS) splenocytes Th2 cytokine IL-4 IL-5 β-actin house keeping gene RT-PCR Th2 cytokine mRNA RT-PCR cytokine ELISA

Th2 cytokine Th2 cytokine mRNA RT-PCR cytokine ELISA

E. hortense 50 IL-4 30 IL-4 30 6 IL-4 30 6 IL-4 30 12 IL-5 30 LPS 250 ng/ml IL-4 30 IL-5 30 6 24 H E. hortense
splenocytes were used with MTT. The splenocytes were treated with 0.5 mg/µl, and 0.3 mg/µl of E. hortense extract.

ELISA was used to detect cytokines in the culture supernatant of splenocytes. The cytokines assayed included IL-4 and IL-5. IL-4 at 9 µg/ml showed increased cytokine secretion. IL-5 at 9 µg/ml also showed increased cytokine secretion. The cytokine levels of IL-4 and IL-5 were measured using ELISA. The cytokine levels of IL-4, IL-5 were increased in mouse splenocytes infected with E. hortense. The cytokines assayed included IL-4 and IL-5.

Keywords: Echinostoma hortense, splenocytes, cytokines, RT-PCR, ELISA
Echinostoma hortense 1926 Asada (1) Tani (2) 1974 Tani (2) 1983 (3) 21 (4)

E. hortense (head worm) (collor spine) (ovary) (testes) (caecum) (1)

1 Radix auricularia (1, 5, 6). 2-3 4 (6-8).

E. hortense (imunoglobulin) (cytokine) (24-28) 19-23 (19-23).

IgE (10-15) (16-18) (24-28) cytokine

- 1 -
(29-34). Cytokines [cytokine] play a key role in regulating T helper (Th) cell responses. Th1 cytokines such as IL-2, IFN-γ, TNF-α, and IFN-γ induce Th1 responses, whereas Th2 cytokines like IL-4, IL-5, and IL-13 induce Th2 responses. Th2 cytokines stimulate B cells to produce IgE isotype switching [isotype switching] (35-38). IgE switching is mediated by IL-4 and IL-5, which contribute to the development of Th2 responses and IgE production. IL-5, in particular, has been shown to promote B cell differentiation into IgE-producing plasma cells (19-23). IL-5 also plays a role in eosinophil activation and Th2 polarization [polarization] (39-42). E. hortense can be a source of cytokines that can induce Th1 or Th2 responses in vitro [in vitro].

- 2 -
1. **Mouse splenocytes**

- Female BALB/c (SPF) mouse
- DMEM (Gibco BRL, Hercules, CA, U.S.A)
- 5 ml syringe (Dongshin Medi-tech, Korea)
- perfusion
- RBC lysis
- RBC lysis 1200 rpm, 5 min
- Splenocytes
- DMEM
- Chamber
- 10% FBS (fetal bovine serum) (Gibco BRL, Hercules, CA, U.S.A)
- DMEM
- Chamber
- 24 hr, 37°C, 5% CO2 chamber (NAPCO 6001, Winchester, VA, U.S.A)
- 1X10^6 cells/mL.

2. **Echinostoma hortense**

- E. hortense
- 0.01 M PBS (phosphate buffered saline)
- 0.01 M PBS
- 100 watt 30 min 30°C
- 5 min 30°C
- 4°C
- 15,000 rpm Lowry (47)
3. *Echinostoma hortense* Mouse splenocytes E. hortense MTT tryphan blue exclusion 5\times 10^4 96 well microplate 100 µl 0 mg/µl, 0.025 mg/µl, 0.05 mg/µl, 0.1 mg/µl, 0.3 mg/µl, 0.5 mg/µl, 0.7 mg/µl, 1 mg/µl DMEM MTT (1 mg/µl, Sigma, St. Louis, MO, U.S.A) 50 µl well 4 \times 1200 rpm 540 nm ELISA Reader (Molecular Devices, CA, U.S.A) 150 µl 540 nm % survival.
4. **Total RNA**

- Lipopolysaccharides (Sigma, St. Louis, MO, U.S.A)
- Splenocytes
- RNA (Intron, Korea)
- Chloroform:isoamylalcohol (Sigma, St. Louis, MO, U.S.A)
- Isopropanol (Merck, Damstadt, Germany)
- DEPC (Sigma, St. Louis, MO, U.S.A)
- D.D.W.
- 1 M NaCl
- TBE buffer (Tris-borate/EDTA electrophoresis buffer, pH 8.0)
- RNA sample
- Loading buffer (Bio-Rad, U.S.A)
- 1% agarose gel (Intron, Korea)
- Polaroid film
- Photo-Documentation Camera (Fisher Scientific, PA, U.S.A)
- 254 nm

5. **Reverse transcription polymerase chain reaction (RT-PCR)**

- Total RNA
- AccuPower RT-PreMix (Bioneer, Korea)
- oligo (dT) (Bioneer, Korea)
- dATP, dGTP, dTTP, dCTP
- 37°C for 10 min
- 42°C for 60 min
- 94°C for 5 min
- cDNA
- PCR
- 5 min
2.5 mM dNTP (Intron, Korea), 4 mM MgCl₂, 10X buffer (50 mM KCl, 10 mM Tris-HCl) (Intron, Korea), 25 mM MgCl₂ (Intron, Korea), primer set 20 pmole/μl (Table 1), Taq polymerase 0.025 U (Intron, Korea), thermal cycler (MJ Research, CA, U.S.A), 5% primer (Bioneer, Korea), 1.5% agarose gel, ethidium bromide (Bio-Rad, U.S.A), 100 V constant voltage, mouse splenocytes, monoclonal anti-mouse IL-4 Ab (R&D system, MN, U.S.A), 250 ng/100μl PBST (phosphate buffered saline), polystyren microplate (Costar, NY, U.S.A), microplate, PBST (phosphate buffered saline).

6. ELISA of cytokines

Mouse splenocytes, 1 ml cytokine dilution (IL-4), sandwich ELISA (R&D System, MN, U.S.A), monoclonal anti-mouse IL-4 Ab (R&D System, MN, U.S.A), 250 ng/100μl PBST, polystyren microplate (Costar, NY, U.S.A), 100 μl cytokine 4°C, well 24, 4°C, 24°C, PBS.
saline-0.05% Tween 20) 5 3 1 1% BSA (bovine serum albumin)/PBS well 200 3 10 90 1% BSA (bovine serum albumin)/PBS well 100 3 10 90 1% BSA (bovine serum albumin)/PBS well 5 3 100. Cytokine well 1% IL-4 cytokine (R&D system, MN, U.S.A) 1 ng/100 18.75 pg/100 1% BSA/PBS well 100 3 100 1% PBST 5 3 100 anti-mouse IL-4 polyclonal Ab (R&D system, MN, U.S.A) 20 ng/100 1% BSA/PBS well 100 3 100 2% PBST 5 3 100 horseradish peroxidase-rabbit anti-goat IgG (Upstate, NY, U.S.A) PBST 1:3000 well 100 3 100 3 7°C 30 5 3 100. OPD (ortho-phenylenediamine) (Sigma, St. Louis, MO, U.S.A) 8 mg 30% H₂O₂ (Fluka, St. Louis, MO, U.S.A) 5 0.1M phosphate citrate buffer (pH 5.0) 12 ml well 100 3 100 2% N H₂SO₄ 100 3 100 2% N H₂SO₄ 100 ELISA (Molecular Devices, CA, U.S.A) 490, 650nm 4°C 24 4°C 24 well 1% polystyren microplate well 4°C 24 4°C 24. microplate PBST (phosphate buffered saline-0.05% Tween 20) 5 3 1 1% BSA (bovine serum albumin)/PBS well 200 3 10 90 1% BSA 5 3 100. anti-mouse IL-5 polyclonal Ab (R&D system, MN, U.S.A) 20 ng/100 1%
1% BSA/PBS 100 μl 24-well plate 96 30 min 1 h
PBST 5 ml 3%  H2O2  horseradish peroxidase-rabbit anti-goat IgG (Upstate, NY, U.S.A) 1:3000 PBST well 100 μl
37°C 30 min 50 μl 30 sec.
OPD (ortho-phenylenediamine) 8 mg 30% H2O: 5 ml
0.1M phosphate citrate buffer (pH 5.0) 12 ml 100 μl
ELISA reader (Molecular Devices, CA, U.S.A) 490, 650 nm.
Table 1. Cytokine primer sequences and amplified fragment sizes used in this study

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primer sequence (5'→3')</th>
<th>Amplified fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>AGG CTG TGC TGT CCC TGT ATC C&lt;br&gt;Sense&lt;br&gt;AGG CTG TGC TGT CCC TGT ATC C&lt;br&gt;Antisense&lt;br&gt;ACC CAA GAA GGA AGG CTG GAA A</td>
<td>395</td>
</tr>
<tr>
<td>IL-4</td>
<td>TCT GTA GAT CAT GGG CAT TTT GAA CGA GGT C&lt;br&gt;Sense&lt;br&gt;TCT GTA GAT CAT GGG CAT TTT GAA CGA GGT C&lt;br&gt;Antisense&lt;br&gt;TGC ATG ATG CTG TTT AGG CTT TCC</td>
<td>306</td>
</tr>
<tr>
<td>IL-5</td>
<td>ATG ACT GTG CCT CTG TGC CTG GAG C&lt;br&gt;Sense&lt;br&gt;ATG ACT GTG CCT CTG TGC CTG GAG C&lt;br&gt;Antisense&lt;br&gt;CTG TTT TTC CTG GAG TAA ACT GGG G</td>
<td>243</td>
</tr>
</tbody>
</table>
1. *Echinostoma hortense* mouse splenocytes

*E. hortense* mouse splenocytes were cultured with MTT solution. Tryphan blue exclusion was performed with 5x10⁴ cells per 96 well microplate well. 100 µL of 0 mg/mL, 1 mg/mL, 2 mg/mL, and 5 mg/mL were added to 24 wells. The MTT solution was 540 nm absorbance. The % survival is shown in Fig. 1. 0.5 mg/mL, 0.3 mg/mL, and 0.1 mg/mL survival rate is shown.
Figure 1. Cytotoxicity of Echinostoma hortense crude antigen and E.S.P. to mouse splenocytes
2. Lipopolysaccharides (LPS) と IL-4, IL-5 mRNA

10% FBS, DMEM, 24時間, 1x10⁶mouse spleenocytes, LPS 250 ng/μl, total RNA, RT-PCR, IL-4, IL-5, IL-4, IL-5, 30分, 30分, 6時間, 24時間 (Fig. 2). LPS と IL-4, IL-5 との mRNA の比較をしました.
Figure 2. Induction of IL-4 and IL-5 mRNA in mouse splenocytes by treatment with lipopolysaccharides (250 ng/mL). Transcription of IL-4 and IL-5 were analyzed after incubating the splenocytes in the presence of various amount of LPS (a) and at various time points (b).
3. *Echinostoma hortense* – IL-4, IL-5 mRNA

10% FBS, DMEM, 24-hour 1x 10^6 mouse splenocytes, 50 u/mL IL-4, IL-5 RNA total splenocytes RT-PCR. Total splenocytes IL-4, IL-5 RT-PCR. Mouse splenocytes 30°C 6 hours. (Fig. 3).
Figure 3. Elavation of IL-4 and IL-5 mRNA in mouse splenocytes by treatment with *Echinostoma hortense* crude antigen (50 μl/mL).

Transcription of IL-4 and IL-5 were analyzed after incubating the splenocytes in the presence of various amount of *Echinostoma hortense* crude antigen (a) and at various time points (b).
4. *Echinostoma hortense* IL-4, IL-5 mRNA

10^6 mouse splenocytes were incubated with 50 μg/mL E. hortense extract for 24 hours. Total RNA was extracted from splenocytes and subjected to RT-PCR. 1x10^6 mouse splenocytes were incubated with IL-4, IL-5 for 30 minutes, cultured for 6 hours, and IL-4, IL-5 for 30 minutes, cultured for 12 hours (Fig. 4). 

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Figure 4. Increase of IL-4 and IL-5 mRNA in mouse splenocytes by treatment with *Echinostoma hortense* E.S.P. (50 μg/mL).

Transcription of IL-4 and IL-5 were analyzed after incubating the splenocytes in the presence of various amount of *Echinostoma hortense* E.S.P. (a) and at various time points (b).
5. ELISA and IL-4 Cytokine Assay

1x 10⁶ mouse splenocytes were incubated in 50 µl of ELISA buffer. ELISA was performed using a sandwich method. After incubation for 36 h, the IL-4 cytokine was assayed by ELISA (Fig. 5).
Figure 5. Expression of IL-4 in mouse splenocytes by treatment with *Echinostoma hortense* crude antigen and E.S.P. (50 μg/μL).
6. ELISA _mouse splenocytes_ IL-5  indirect method

1x 10⁶ mouse splenocytes in 50 μ/l, IL-5 50 μ/l. ELISA using indirect method, 30, 36, 48, and 96 h. IL-5 stimulates 1x 10⁶ mouse splenocytes. 36 h, 48 h, and 96 h (Fig. 6).
Figure 6. Expression of IL-5 in mouse splenocytes by treatment with *Echinostoma hortense* crude antigen and E.S.P. (50 μg/μL).
Echinostoma hortense\textsuperscript{(4)}

cytokine\textsuperscript{(4)}
IgE (10-15)\textsuperscript{(4)}
eosinophil (16-18)\textsuperscript{(4)}
Th2 cytokine\textsuperscript{(4)}
IL-4\textsuperscript{(4)}
IL-5\textsuperscript{(4)}

IL-4\textsuperscript{(4)}
B cell\textsuperscript{(4)}

IL-4\textsuperscript{(4)}

IL-5\textsuperscript{(4)}

IL-4\textsuperscript{(4)}

IL-5\textsuperscript{(4)}

Schistosoma mansoni\textsuperscript{(wurm or egg)}
splenocytes\textsuperscript{(49)}

Th2 cytokine\textsuperscript{(49)}

Th1 cytokine\textsuperscript{(49)}

Ag\textsuperscript{(49)}

CD4\textsuperscript{+} cell\textsuperscript{(49)}

Th1 cytokine\textsuperscript{(49)}

IL-4\textsuperscript{(49)}

Leishmania major\textsuperscript{(in vitro)}

E. hortense\textsuperscript{(RT-PCR)}
mRNA\textsuperscript{(30)}

IL-4\textsuperscript{(30)}

IL-4\textsuperscript{(30)}

IL-4\textsuperscript{(30)}

IL-4\textsuperscript{(30)}

Leishmania major\textsuperscript{(51)}

E. hortense\textsuperscript{(RT-PCR)}
mRNA\textsuperscript{(30)}

- 22 -
IL-5 reduced to 30ºC for the first 6 hours (Figure 3, 4). ±â»ýÃæÀÇ
Shistosoma mansoni rat splenocytes IL-4 IL-5 mRNA expression was
Shistosoma mansoni rat splenocytes IL-4 IL-5 mRNA expression was
IL-4 cytokine expression IL-4 IL-5 (52), alveolar
echinococcosis® peripheral blood mononuclear cells E.
multilocularis® cytokine expression IL-4 IL-5 cytokine expression IL-4 IL-5 mRNA expression IL-4 IL-5 cytokine expression IL-4 IL-5 mRNA expression IL-4 IL-5 cytokine expression IL-4 IL-5 mRNA expression IL-4 IL-5 cytokine expression IL-4 IL-5 mRNA expression IL-4 IL-5 cytokine expression IL-4 IL-5 mRNA expression (53).
heat-inactivated Brucella abortus®
CD4’ CD8’ T cells cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression (Figure 5, 6).

E. hortense® cytokine mRNA expression ELISA
IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression IL-4 IL-5 cytokine mRNA expression (Figure 5, 6).

- 23 -
LPS-stimulated murine macrophage 

Spionetra erinaceieuropa 

Th1 cytokine tumor necrosis factor-α (55).

IL-4, IL-5 are plays a role in cytokine ELISA analysis. ELISA results for cytokine IL-4, IL-5 in murine macrophage. 

ELISA and RT-PCR analysis for cytokine IL-4, IL-5 in murine macrophage. 

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ELISA and RT-PCR analysis for cytokine IL-4, IL-5 in murine macrophage.
*Echinostoma hortense* (crude antigen) (excretory-secretory product; E.S.P.) mouse splenocytes total RNA RT-PCR IL-4, IL-5 mRNA cytokine ELISA IL-4, IL-5

1. MTT E. hortense Effect E. hortense 0.5 mg/µL, mouse splenocytes 0.3 mg/µL

2. LPS (250 ng/µL) mouse splenocytes IL-4, IL-5 mRNA

3. *E. hortense* (50 µg/µL) mouse splenocytes IL-4 mRNA

- 25 -
4. *E. hortense* (50 / /) mouse splenocytes IL-4 mRNA 30° 12° 4°, IL-5 mRNA 30° 12° 4°.

5. cytokine IL-4 9° 3° 9°, IL-5 9° 3° 9°. cytokine IL-4, IL-5 cytokine.

6. cytokine IL-4, IL-5 36° 3° 9°.


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Abstract

Profiles of Th2 Cytokine Expression in Mouse Splenocytes after Stimulation with Echinostoma hortense Crude Antigen and Excretory-Secretory Product

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The Graduate School
Yonsei University

In this study, 6 week-old female BALB/c mice were used to obtain splenocytes. This splenocytes were examined for the Th2 cytokine expression after treatment with Echinostoma hortense crude antigen, E.S.P. and lipopolysaccharide by time-dependent assay. Among the Th2 cytokines, IL-4 and IL-5 were selected and β-actin was used to house keeping gene during RT-PCR. RT-PCR technique was used to detect Th2 cytokine mRNA expression in splenocytes and ELISA was used to examine the concentration of increased cytokine in splenocytes culture supernatant.
The result of Th2 cytokine mRNA expression with RT-PCR, in the cases of IL-4 and IL-5 with treated by crude antigen (50 μg/ml), IL-4 mRNA expression was peak at 30 min and continued to 1 hour, and IL-5 mRNA expression was peak at 30 min and continued to 6 hours. And another case of IL-4 and IL-5 with treated by E.S.P. (50 μg/ml), IL-4 mRNA expression was peak at 30 min and continued to 6 hours, and IL-5 mRNA expression was peak at 30 min and continued to 12 hours. The last case of IL-4 and IL-5 with stimulation by LPS (250 ng/ml), IL-4 mRNA expression was peak at 30 min and continued to 3 hours, and IL-5 mRNA expression was peak at 30 min and 6 hours, and continued to 24 hours. MTT assay was used to examine cytotoxic effect on mouse splenocytes of crude antigen and E.S.P. These stimulator did not have cytotoxicity to mouse splenocyte. In this case, the result of cytotoxicity was converted to survival rate of percentage and the optimal doses of crude antigen and E.S.P. were 0.3 mg/ml and 0.5 mg/ml.

To detect the dose of increased cytokine in culture supernatant, ELISA method was used. The consequence of IL-4 and IL-5 with activation by crude antigen, IL-4 cytokine was more induced in 9 hours than IL-5. In other case of IL-4 and IL-5 with activation by E.S.P., both of IL-4 and IL-5 was not increased in 1 hour to 36 hours.

In conclusion, *E. hortense* crude antigen might have play the roles of stimulator to mouse splenocytes and IL-4 was more induced than IL-5 during stimulation with crude antigen.

Key words: *Echinostoma hortense*, crude antigen, E.S.P., splenocytes, RT-PCR, ELISA