Preventive and Therapeutic Effects of *Anisakis simplex* Larval Protein in a Mouse Model of Crohn'S Disease

Hee-Jae Cha, Mee Sun Ock

Department of Parasitology and Genetics, College of Medicine, Kosin University, Busan, Korea

아니사키스 유충 유래 단백질의 크론병 예방 및 치료 효과

차희재·옥미선

고신대학교 의과대학 기생충학·유전학교실

Objectives: Some helminths have been known to have a treatment effect in inflammatory bowel diseases, including Crohn's disease (CD); however, live parasite therapy can cause unwanted side effects. To develop a safe therapeutic, we investigated the preventive or therapeutic potential of proteins from the third stage larva of *A. simplex* in a mouse model. We also analyzed the cytokine profile from splenic and mesenteric lymph node lymphocytes to elucidate the underlying immunological mechanism.

Methods: CD was induced in mice with DSS, and the effect of an *A. simplex* larval protein on CD was assessed. A change in body weight and DAI (disease activity index) were observed in mice. The expression levels of cytokines from mesenteric lymph nodes (MLN) compared to splenic lymphocytes were measured with ELISA.

Results: Peritoneal administration of preventive and therapeutic *A. simplex* larval proteins attenuated DSS-induced CD by a reduction of the DAI and weight loss. A shortening of colon length was more definitely observed in the therapeutic group than in the preventive group. The cytokine expression levels were more obvious in lymphocytes from mesenteric lymph nodes than from splenic lymphocytes.

Conclusions: Taken together, these results suggest that *A. simplex* proteins can change cytokine profiles and may have a preventive effect in DSS-induced CD mice.

Key Words: *Anisakis simplex*, Crohn's disease, Cytokine, Prevention

Inflammatory bowel diseases (IBD) such as Crohn's disease (CD) are chronic inflammatory disorders of the intestine. Although the pathogenesis of IBD remains unknown, it is now considered to be a multifactorial disease that involves both genetic and environmental factors. The hygiene hypothesis (Strachan, 1989) contends that infants and children who lack exposure to infectious agents, symbiotic microorganisms, and parasites are more susceptible to allergic diseases by suppressing the natural development of the immune system.[1] The high prevalence of allergic diseases in developed countries compared to less developed countries strongly supports the theory of the "hygiene hypothesis." Improved hygiene has been considered to alter the balance between type 1 (Th1) and type 2 (Th2) immune responses due to

Corresponding Author: Mee Sun Ock, Department of Parasitology and Genetics, College of Medicine, Kosin University, 262 Kamchunro Seogu, Busan, 602-703, Korea
TEL: +82-51-990-6424 FAX: +82-51-990-3081 E-mail: sunnyock@kosin.ac.kr

a failure of immune regulation resulting in allergy-mediated Th2 responses.[2] In the absence of Th1-polarizing stimuli in childhood, mucosal immune responses fail to overcome their inherent Th2 bias and instead become slanted in the direction of allergy later in life. Helminths can also interact with both innate and adoptive immunity in the host and the resulting stimulation of a regulatory immune response.[3] Thus, exposure to helminths may help prevent or even ameliorate Crohn's disease.

Helminth therapies against IBD are being tried with various parasites. Summers et al. (2006) have successfully utilized the pig whipworm (*Trichuris suis*) in the treatment of patients with active Crohn's disease.[4] Mortimer et al. (2006) also found that infection with *Necator americanus* larvae is promising for use in preliminary clinical therapeutic trials of asthma.[5]

However, helminth therapies have raised some concerns about the safety of living parasites. *T. suis* larvae can wander aimlessly, escaping from the gut of humans.[6] Hsu et al. (2005) were also concerned about the detrimental effects of treatment with *T. suis* ova in nonresponsive patients.[7]

Therefore, identification and characterization of immunologically active proteins from helminths might ensure safety and overcome the revulsion to this therapy, as some patients are reluctant to ingest living parasites.

Anisakis simplex excretory-secretory protein was proven to induce the production of proinflammatory cytokines and chemokines from a mouse lung epithelial cell line and primary lung epithelial cells.[8] Further, the recombinant MIFs (macrophage migratory inhibition factor) from *A. simplex* third stage larvae suppressed allergic airway inflammation by increasing TGF-β and IL-10.[9] These reports imply that *A. simplex*-derived proteins induce cytokines and can have a beneficial effect against CD.

The aim of this study was to investigate whether crude extract and ES product from *A. simplex* third stage larvae have a preventive or therapeutic potential in DSS-induced CD mice. We also examined the underlying immunological mechanism of the beneficial effect of *A. simplex* proteins by analyzing the cytokine profile of splenic and mesenteric lymph node lymphocytes.

MATERIALS AND METHODS

1. Mice

Eight-week-old female C57BL/6 mice were purchased from Hyochang Science (Daegu, Korea). The mice were housed in a laminar flow cabinet throughout the experiments and were allowed free access to standard rodent chow. All animal studies were approved by the Animal Care and Use Committee of the Kosin University College of Medicine.

2. *Anisakis simplex* larvae collection and excretory-secretory product and somatic product preparation

A. simplex L3 larvae were collected manually from the viscera, flesh, and body cavities of naturally infected mackerels (*Scomber japonicus*) and thoroughly washed with PBS (phosphate buffered saline). The preparation of somatic product (SP) and excretory-secretory product (ES) from *A. simplex* L3 larvae was performed as described previously.[10] Briefly, about 1 g (300 larvae) of larvae was frozen in liquid nitrogen and smashed by mortar. To extract the proper amounts of SP proteins, the protein extraction solution was added and stored on ice for

30 min according to the manufacturer's instructions (PRO_PREP^{TM}, iNtRON Biotechnology, Seoul, Korea). The supernatant was collected after centrifugation at maximum speed for 10 min at 4°C. The amount of protein was measured by the Bradford method. To prepare the ES product, Larvae were incubated in DMEM (Dulbecco's Modified Eagle Medium) with gentamycin (150 mg/mL) and vancomycin (10 mg/mL) at 0.5 mL/larva and 37°C for 48 hr. The media were collected and centrifuged at 2500 rpm for 20 min and concentrated with Amicon stirred cells with a cut off molecular weight of less than 10,000 (Millipore Corp, Massachusetts, USA).

3. Experimental groups of mice and *A. simplex* protein injection

We divided the mice into 4 groups, which consisted of 8 mice in each group. One experimental group of mice (preventive group) was injected with SP and ES products intraperitoneally before DSS solution was administered. The other group of mice was first given DSS solution for 4 days and then treated with SP and ES product (therapeutic group). The amount of protein injected in both groups of mice was 10 μg a day for 7 days. Mice were checked each day for morbidity, and their weights were recorded. The negative control group of mice received only drinking tap water. The positive control group was given DSS solution only.

4. Induction and assessment of DSS-induced colitis

In order to induce intestinal inflammation, mice were given a solution of drinking water containing 3% DSS (36-50 kDa; MP Biomedicals, Santa Ana, CA, USA) for 7 days ad libitum. The DSS solution was replenished every other day, and at the end of the fourth day, the

animals were sacrificed. The assessment of disease activity index (DAI) was carried out once a day. DAI was the combined score of weight loss (scored as: 0, none; 1, 1-5%; 2, 5-10%; 3, 10-20%; 4, over 20%), stool consistency (scored as: 0, well-formed pellets; 2, loose stools; 4, diarrhea) and bleeding (scored as: 0, negative hemoccult; 2, positive hemoccult; 4, rectal bleeding). The colons were removed and measured from 1 cm above the anus to the top of the cecum.

5. Lymphocyte preparation and cytokine analysis

After the mice were sacrificed, lymphocytes from their spleens and mesenteric lymph nodes (MLN) were isolated to determine the level of specific cytokines. The spleens and MLN were disrupted and treated with ACK hypotonic lysis solution (Sigma, Seoul, Korea) for 2 min at room temperature for RBC (red blood cell) lysis. RBC-depleted lymphocytes were filtered through 100 μm mesh (Small Parts Inc., Seattle, WA, USA), and the cells were plated in wells at 5 x10^6 cells/mL in RPMI 1640 with 10% fetal bovine serum (FBS) and penicillin / streptomycin. The plated cells were then incubated for 72 h at 37°C in 5% CO_2. After 72 h incubation, the supernatants were harvested. The cytokine levels, including IL-1α, IL-1β, IL-2, and IFN-γ, were determined with ELISA in accordance with the manufacturer's instructions (eBioscience, R&D Systems, Korea). The absorbance of the final reactant was determined at 450 nm using an ELISA plate reader.

6. Statistical analysis

Experiments were conducted at least in triplicate, and data are expressed as means $\pm$ SD. Student's t-test was applied for comparison between groups. *P* values of < 0.05 were considered statistically significant in

all experiments.

RESULTS

1. A challenge with *Anisakis simplex*-derived protein can protect mice against DSS-induced colitis

To evaluate the potential effects of *A. simplex*-derived protein, the mice were treated with SP and ES products (10 μg/mouse, once a day) for 7 days before or after DSS administration. A remarkable weight loss of mice after DSS administration was observed. The body weight in the preventive group slowly increased until the 12th day and decreased after that. This reduction was consistent with the DSS injection. In the therapeutic group, the body weight rapidly fell after DSS administration, but the mice gained weight along with injection of *A. simplex* protein (Fig. 1). The average body weight was higher in the preventive group than in the therapeutic group (18.3 g ± 1.22, 18.0 g ± 1.14). The DAI decreased after 14 days in the preventive group, whereas it was
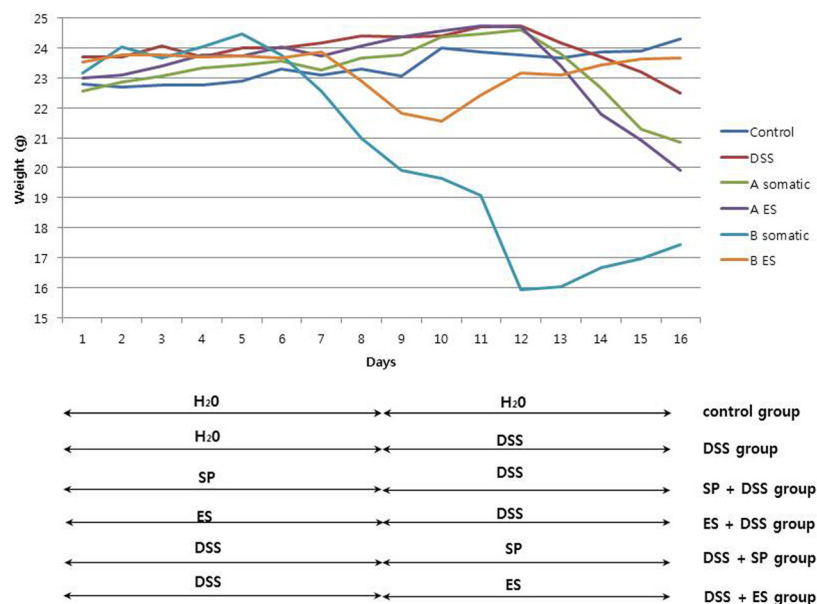

Fig. 1. Changes in body weight in each group of mice. SP + DSS and ES + DSS groups are preventive groups; DSS + SP and DSS + ES groups are therapeutic groups. The average body weight was higher in the preventive groups than in the therapeutic groups.
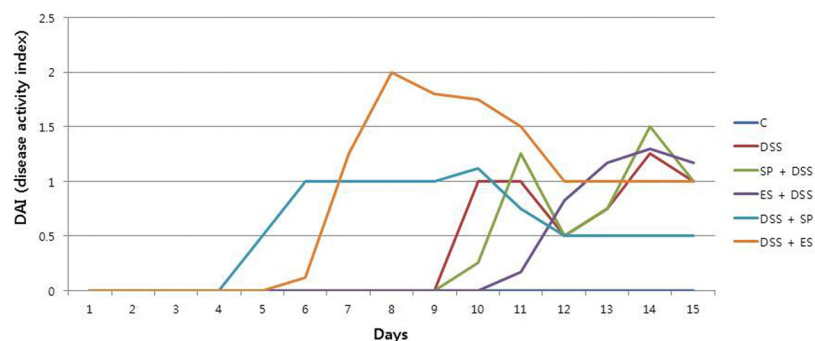

Fig. 2. DAI's were measured daily during the experimental period in each group of mice. SP + DSS and ES + DSS groups are preventive groups; DSS + SP and DSS + ES group are therapeutic groups.

sustained in the therapeutic group (Fig. 2). The colon length of mice in the therapeutic and DSS groups was much shorter than in the preventive and negative control groups (Fig. 3). These findings imply that *A. simplex* protein injection resulted in a reduction of the DAI and maintenance of body weight and colon length, especially in the preventive group.

2. Proteins from *Anisakis simplex* L3 can change the cytokine profile in DSS-treated mice

The cytokine expression levels were obvious in lymphocytes from mesenteric lymph nodes compared to splenic lymphocytes (Fig. 4). The pro-inflammatory cytokines IL-1α and IL-1β were highly increased in the MLN of the DSS-injected mice group. A decrease in IL-1α was evident in the preventive group, while the expression level of IL-1β dropped

in both the preventive and therapeutic groups (Fig. 4A, B). These results indicate that *A. simplex*-derived

Fig. 3. The representative colon from each group of mice. (C) control group, (D) DSS-treated group, (A) preventive group, (B) therapeutic group. The colons of the mice in the preventive group maintained their original length.
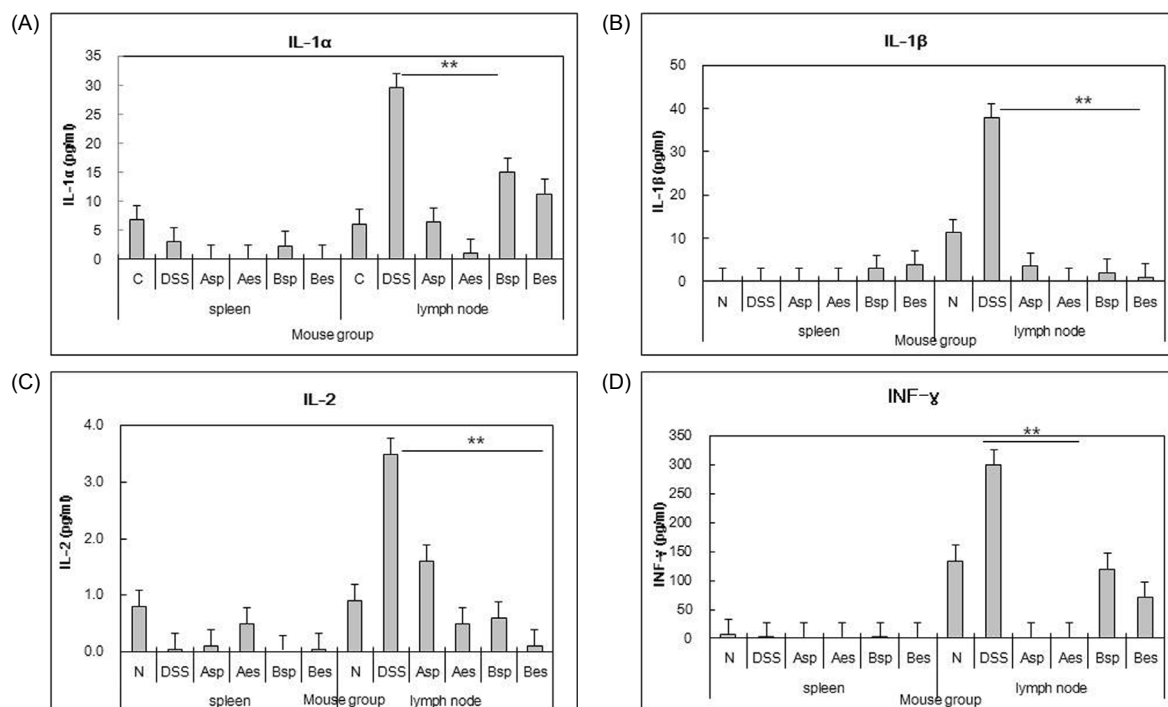

Fig. 4. Cytokine profile of splenic and lymph node lymphocytes. Expression levels of IL-α (A), IL-1β (B), IL-2 (C), and IFN-γ (D) were measured with ELISA after administration of *A. simplex* protein. C; control, DSS; DSS only, Asp and Aes denotes preventive group injected with SP (somatic protein) and ES (excretory-secretory) protein.

protein can suppress the expression of proinflammatory cytokines. In the case of IL-2, the decrease was noticeable in the therapeutic group, particularly by ES product (Fig. 4C). IFN-γ was nearly unexpressed in the spleen, while the expression of IFN-γ in MLN lymphocytes of the DSS group was significantly increased (Fig. 4D). The injection of SP and ES nullified the production of IFN-γ. This effect was more prominent in the preventive group. These results suggest that protein from *A. simplex* can alter the cytokine profile and improve the condition of mice suffering from DSS-induced colitis.

DISCUSSION

The cytokine profiles in IBD are the key pathophysiologic factors in determining the initiation and severity of the disease. In this work, we investigated whether *Anisakis* larval proteins can change the pattern of cytokine expression and have a preventive or therapeutic effect in a mouse CD model. We found that MLN lymphocytes have stronger responses than splenic lymphocytes. It was also revealed that *Anisakis*-derived proteins can mitigate the disease by decreasing proinflammatory cytokine production and maintaining the length of the colon. ES proteins induced a more robust inhibition of cytokine production compared to SP proteins. The expression of IL-1α, IL-1β, and IFN-γ were significantly downregulated in the preventive groups, while the IL-2 level was considerably decreased in the therapeutic groups. It is therefore reasonable to deduce that *A. simplex* larval protein can be a potential agent for improving CD in a preventive manner.

CD has been characterized as a Th1 cytokine-mediated disease accompanied by the production of interferon (IFN)-γ.[11-12] Therefore, IFN-γ was tried as a therapeutic agent for CD.[13-14] When we applied *Anisakis* proteins in a CD mouse model, the amount of IFN-γ expression significantly reduced, especially in the preventive groups. IFN-γ also affects downstream effector cells to induce the production of pro-inflammatory cytokines such as IL-1β. The level of IL-1β has more than tripled in the DSS group compared to the control group. The injection of *Anisakis* proteins lowered the level of IL-1β to below the level of the control group. The expression level of IL-1α also decreased, although the reduction was less noticeable than for IL-1β. IL-2 showed a significant decrease in the therapeutic group, especially in the ES protein-injected group. These results show that *A. simplex* larval proteins can bring a favorable effect in a mouse model of CD. This favorable effect was reaffirmed by comparing colon length between the mice of the DSS and protein-injected groups.

Many parasites proteins were proven to ameliorate IBD in human and animal models. Soluble proteins from *Schistosoma mansoni* and *Ancylostoma caninum* showed a therapeutic effect in a TNBS-induced colitis mouse model by decreasing IFN-γ and IL-17 in colonic tissue and mesenteric lymph nodes.[15] In addition, *T. suis* egg therapy has already successfully been tried in CD patients.[16] *Trichinella spiralis* infection brought a similar protective effect in DNBS—induced colitis mice, which was associated with an IFN-γ and IL-12 decrease.[17] These previous studies correspond with our result of a significant reduction of IFN-γ. This finding leads to the conclusion that helminth or helminth-derived proteins could be used in the treatment and prevention of IBD.

In conclusion, *A. simplex* proteins can exert a treatment effect by decreasing proinflammatory cytokines and can be a candidate for shaping the human immune system. These trials can help us understand the mechanism of CD and provide a new insight for CD control.

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