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Cytokine-Inducing and T Cell Mitogenic Effects of *Cordyceps hepialidicola*

Jong-Soon Lim[1,3], Seung-Hyung Kim[1,3], Jeong-Youl Choi[2,3], Jin-Seo Park[2,3], Seong Joo Park[2,3], and Kwang-Soo Shin[2,3]*

[1]*College of Oriental Medicine,*
[2]*Department of Microbiology, College of Sciences, and*
[3]*Institute of Traditional Medicine and Bioscience, Daejeon University, Daejeon 300-716, Korea*

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The morphological characteristics of newly isolated *Cordyceps hepialidicola* were characterized, and the phylogenetic relationships with other *Cordyceps* species were investigated using a sequence analysis of the internal transcribed spacer (ITS). The PCR product of 592 bp showed a homology of 92 and 91% with *C. militaris* and *C. nutans*, respectively. In an *in vitro* model using mouse peripheral blood mononuclear cells (PBMC), a methanol extract of *C. hepialidicola* induced multiple cytokines, including IFN-γ, IL-4, and IL-18. The extract also enhanced the percentages of the $CD4^+$ and $CD8^+$ T cells in the healthy murine PBMCs to 56.1% and 13.0%, respectively. The percentages of $CD4^+$and $CD8^+$ in the untreated controls were 28.4 and 7.3%, and concanavalin A-treated positive controls were 62.4 and 18.3%, respectively.

Key words: *Cordyceps hepialidicola*, taxonomy, cytokines, T lymphocytes

Cordyceps are natural fungi that grow on various insect species during the winter. In spring, the fungi fall from the insect surface and are ready for harvesting. There are approximately 300 species of these fungi reported in Korea according to their morphological characteristics (11). *Cordyceps* are well-known and important ingredients in traditional Oriental medicine. In particular, *C. sinensis* is widely used in hospitals both as a clinical medicine and as a household remedy. The effects of this fungus are claimed to include preventing and reducing inflammation, eliminating coughs, eliminating phlegm, calming emotions, improving shortness of breath, stimulating sexual functions, lowering blood cholesterol, increasing the supply of blood to the body, and modulating the general functioning of the cellular system. *Cordyceps* also can strengthen the body's immunity, and treat other conditions resulting from insufficient vitality and compromised immune functions. Kuo *et al.* (7) reported that the methanol extract of the *C. sinensis* fruit body exhibits an inhibitory effect on the growth of K562, Vero, Wish, Calu-1, and Raji tumor cell lines. These inhibitory activities are not due to polysaccharides or cordycepins. Therefore, it has been suggested that other tumor cell growth inhibitors may be contained in *C. sinensis*. Recently, it has been demonstrated that an aqueous extract of the nonsexual stage of *Cordyceps*, *Paecilomyces japonica*, shows cytotoxicity against various tumor cells by apoptosis induction (9).

The present paper describes the taxonomical characteristics of a newly isolated *Cordyceps hepialidicola* and the effects of this fungus on the regulation of cytokine mRNA expression and T cell activation, which are also compared to those of *C. sinensis* and *Beauveria bassiana*.

* To whom correspondence should be addressed.
(Tel) 82-42-280-2439; (Fax) 82-42-280-2608
(E-mail) shinks@dragon.taejon.ac.kr

Materials and Methods

Microorganisms and extract preparation

The fruit body of *C. hepialidicola* was obtained from the Fungal Culture Collection at Daejeon University (FCCDU), and the *C. sinensis* and *B. bassiana* used are commercially available. Methanol (70%, v/v) was added to each fungus and the mixture incubated for 3 h. The resulting extract was concentrated and lyophilized.

Amplification and sequencing of the ITS region

The ITS region of *C. hepialidicola* was amplified with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (TCCTCCGCTTATTGATATGC) (15). The amplification was performed in a 50 μl reaction mixture containing 50 pmol of the primers, 5 units of *Taq* polymerase, 2.5 mM of a dNTP mixture, 5 μl of a 10×PCR buffer, and 10 ng of template DNA. The first cycle consisting of a 3 min denaturation at 94°C was followed by 35 cycles of 30

s at 94°C, 30 s at 49°C, and 1 min at 72°C, with a final extension of 10 min at 72°C. The PCR products were analyzed by electrophoresis with 1% (w/v) agarose and purified using DNA PrepMate™ (Bioneer, Korea) according to the procedure recommended by the supplier. The purified PCR product was sequenced using an Automated DNA Sequencer (ABI PRISM™ 377, Perkin Elmer). Homologous sequences were found by a BLAST Search. The ITS sequence determined in this study was deposited in the GenBank under accession number AF315649.

Preparation of peripheral blood mononuclear cells (PBMC)

The PBMC were isolated from the splenic cells of Balb/c mice by Histopaque-1077 density gradient centrifugation (3) and cultured in an RPMI 1640 medium containing 10% (v/v) fetal bovine serum (FBS) for 1 h. Following the treatment of each fungal extract (10 or 100 μg/ml), the culture was further incubated at 37°C for 3 h.

RT-PCR for the analysis of cytokines expression

For the denaturating reaction, 3 μg of total RNA was heated at 75°C for 5 min and then slowly cooled. The RT reaction (20 μl) was initiated by the addition of 200 U of Moloney murine leukemia virus reverse transcriptase (Promega), 2.5 μl of 10 mM dNTPs, 1 pmol of random sequence hexanucleotides, 20 U of RNase inhibitor, 1 μl of 100 mM DTT, and 4.5 μl of a 5×RT buffer, followed by extensions at 37°C for 60 min and 95°C for 5 min. The reverse transcribed mixture (3 μl) was subjected to a PCR in a 30 μl mixture (3 μl of a 10×PCR buffer, 3 μl of 2.5 mM dNTPs, 1.0 U of *Taq* polymerase, and 10 pmol of each primer). The primer sequences used in this study are described in Table 1. The PCR temperature program was 95°C for 5 min, 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 25 cycles and with a final 3 min extension at 72°C. Following amplification, 20 μl of each product was analyzed by electrophoresis in a 1.2% (w/v) agarose gel and stained with ethidium bromide.

Flow cytometry analysis

Approximately 1×10^6 PBMC cells were transferred to a 24-well plate and incubated for 72 h. The extract of each fungus (100 μg/ml) and concanavalin A (10 μg/ml) were added to the culture. After the reaction, the reaction mixtures were placed on ice and immunofluorescence staining was performed. Phycoerythrin (PE)-anti-CD3e, fluorescein isothiocyanate (FITC)-anti-CD4, and FITC-anti-CD8 were added, then the new mixtures were incubated on ice for 30 min. The resulting cells were washed with a PBS buffer 3 times and analyzed using a Flow cytometer (Becton Dickinson).

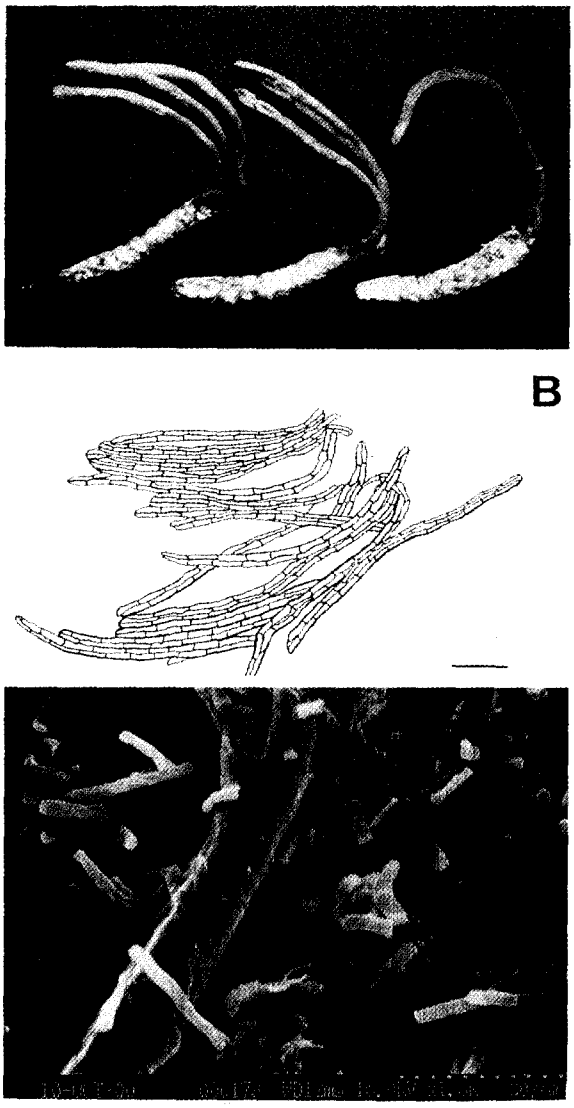

Fig. 1. Morphological characteristics of *C. hepialidicola*. (A) Photograph of fruit body; (B) Fine structure of asci with a scale bar indicating 10 μm; (C) SEM photograph of ascospore.

Table 1. Oligonucleotide primers for cytokine mRNA amplification

Cytokine	Sense primer	Antisense primer	PCR product size
IL-4	ATGAACTCCTTCTCCACAAGCGC	GAAGAGCCCTCAGGCTGGACTG	205 bp
IL-10	ATGGCCTAGTCAGTCTCTAAAT	GTCACAGTCAGCTGTATAGGG	242 bp
IL-12 (p35)	GCTCCTTCAGGAATCTGTTC	GGCTCATGTACTTTCATGAG	289 bp
IL-12 (p40)	ATCTGCTGCTCCACAAGAAG	TGATGAAGAAGCTGGTGCTG	301 bp
IL-18	ACTGTACAACCGCAGTAATAC	AGTGAACATTACCGCTTTATC	540 bp
INF-γ	AGCGGCTGACTGAACTCAGATTGTAG	GTCACAGTTTTCAGCTGTATAGGG	405 bp

Results and Discussion

Taxonomy of C. hepialidicola

As shown in Fig. 1A, the fungus formed singly or in a small group on the larva of *Endoclyta excrescens*. The fruit body was 3-10×0.12-0.2 cm and the slightly swollen fertile head had a finely roughened surface and tapered into a sterile slender stalk. The asci were very long, 1.5× 43.3 μm (Fig. 1B) and the ascospores were seen breaking up into partial spores. The size of the spores was 1-2×7-8 μm (Fig. 1C). The size of the PCR product from *C. hepialidicola* was 529 bp and the sequence analysis of the ITS region showed a high G+C content (50%). The secondary structure observed in the ITS region of the powdery mildew fungi was supported by a high G+C content (12). It has already been demonstrated that ITS regions as well as the rDNA coding regions form secondary structures, which play a role in the maturation of rRNA precursor (10). The nucleotide sequences were then compared with the sequences available in the GenBank databases using the BLAST program. When comparing the ITS sequence from *C. hepialidicola* with those from *C. nutans* and *C. militaris*, the sequences were shown to have a 92 and 91% identity, respectively. However, a low similarity with the sequence from *C. sinensis* was shown, even though the host specificity was the same. In the unrooted Neighbor-Joining tree of the ITS regions, *C. hepialidicola* was found to be closely related to *C. militaris*, *C. scarabaeicola*, *C. nutans*, and *Isaria japonica* (Fig. 2).

Cytokine mRNA expression

When the PBMC were treated with methanol extracts of *C. sinensis*, *B. bassiana*, and *C. hepialidicola*, the expression of the cytokine mRNAs, IL-4, IL-18, and IFN-γ, was increased in a dose-dependent manner (Fig. 3). The expression of IL-12 p35 mRNA by the PBMCs was slightly increased by the fungal extracts. IL-12 forms the heterodimeric molecules of 70 kDa that are composed of two disulfide bond-linked glycosylated chains of 40 kDa and 35 kDa. The p35 gene is constitutively expressed in most cell types but the regulated expression of p40 is found in functional IL-12 producing cells (5, 16). IL-12 stimulates the proliferation and cytotoxic activity of T cells and NK cells, and induces the production of cytokines, particularly IFN-γ (1, 14). IL-10 can be expressed by a variety of cells, usually in response to an activation stimulus and its transcription can be regulated by transcription factors Sp1 and Sp3, which are expressed constitutively by many different cell types (4, 6, 13). IL-10, together with IL-4, is mostly produced by Th2 cells. It induces the differentiation of Th2 cells and inhibits the production of Th1 type cytokines including IFN-γ and IL-12 (6). IFN-γ is produced by macrophages, T cells, and NK cells in response to mitogenic or antigenic stimulation and inhibits the prolifer-

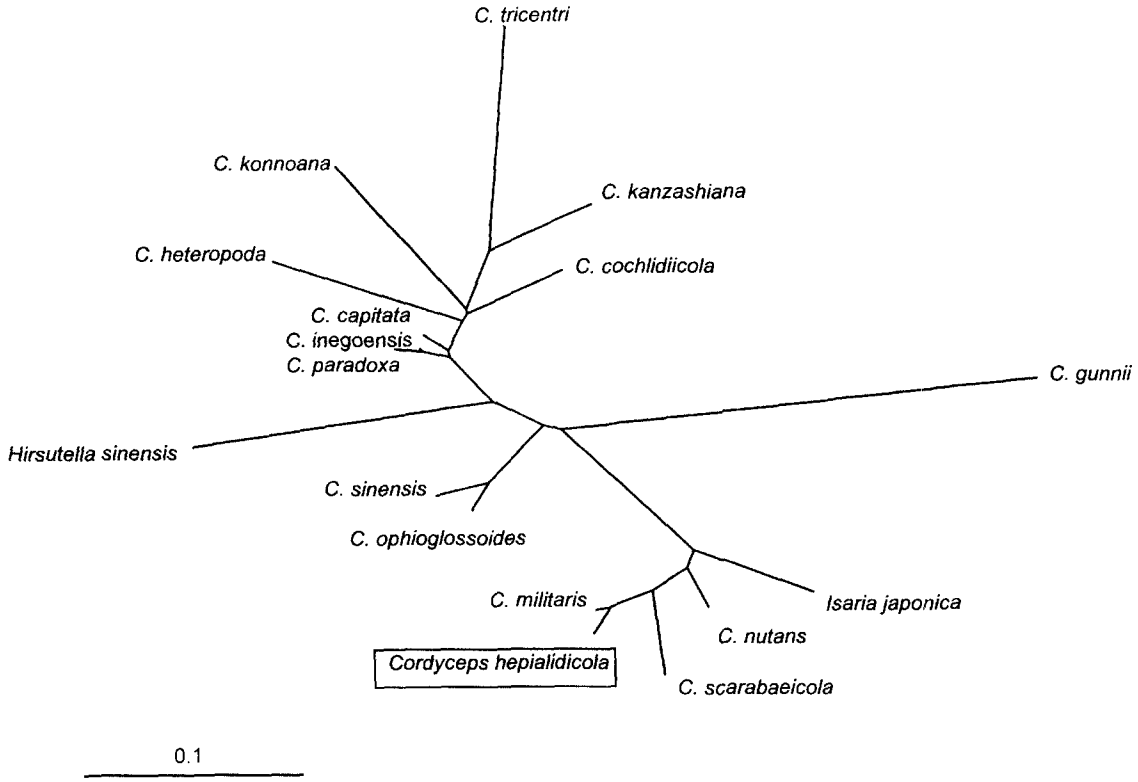

Fig. 2. Unrooted Neighbor-Joining tree from analysis of ITS regions of *Cordyceps*. The scale bar indicates the distance of 0.1 unit.

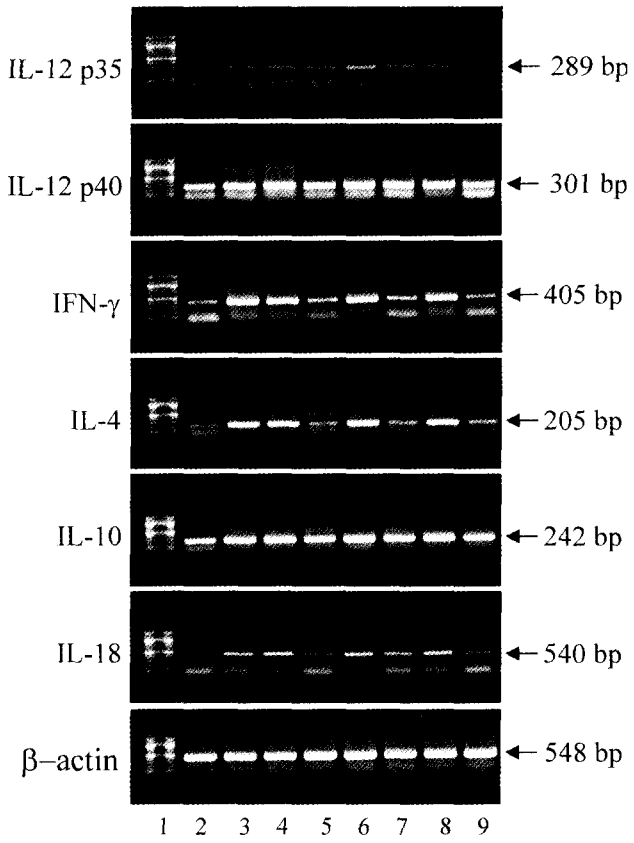

Fig. 3. Effects of fungal extract on cytokines mRNA expression in mouse PMBCs. The PBMCs were pretreated with the fungal extracts and Concanavalin A (10 μg/ml) for 3 h. The amplified PCR products were electrophoresed on a 1.2% agarose gel. Lane 1, 100 bp DNA markers; lane 2, RPMI-1640 media control; lane 3, concanavalin A; lane 4, *C. sinensis* (100 μg/ml); lane 5, *C. sinensis* (10 μg/ml); lane 6, *B. bassiana* (100 μg/ml); lane 7, *B. bassiana* (10 μg/ml); lane 8, *C. hepialidicola* (100 μg/ml); lane 9, *C. hepialidicola* (10 μg/ml).

ation of Th2 cells (2). The cytokine profile of these results does not show the polarization of Th1/Th2 balance but the transcription of cytokine mRNAs in PMBCs treated with fungal extracts is augmented significantly (Fig. 3). IL-18 has an IFN-γ inducing activity in T cells and NK cells in the presence of IL-12 and upregulates the cytotoxic activities of NK and $CD8^+$ T cells (8). From these results, it is suggested that extract of *C. hepialidicola* fruit body contains the factors that induce the expression of various cytokines. Additionally, the activity of the immune system can be augmented by these factors.

Activation of T cells

Fig. 4 shows the number of $CD4^+$ T cells in the PBMCs treated with each fungal extract. The subpopulation of $CD3e^+/CD4^+$ in the untreated and concanavalin A-treated controls were 28. 4 and 62.4%, respectively. The $CD3e^+/CD4^+$ cell subpopulations for the extract of *C. sinensis*, *B. bassiana*, and *C. hepialidicola* were 54.0, 58.4, and 56.1%,

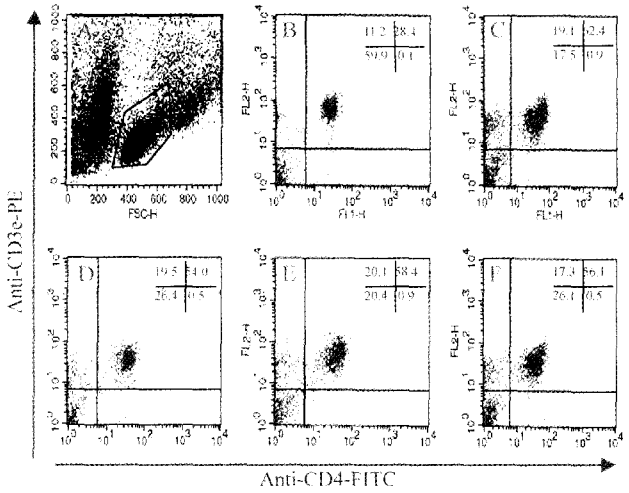

Fig. 4. Effects of fungal extract on proliferation of $CD3e^+$ and $CD4^+$ in PBMCs of healthy murine. The PBMCs were isolated from Balb/c mice and cultured in the absence or presence of a fungal extract (each 100 μg/ml) for 72 h and analyzed using a flow cytometer. The events in panel (A) were backscattered onto a dot-plot of SSC versus FSC and CD3e-PE versus CD4-FITC (B to F). B, no treatment; C, concanavalin A; D, *C. sinensis*; E, *B. bassiana*; F, *C. hepialidicola*.

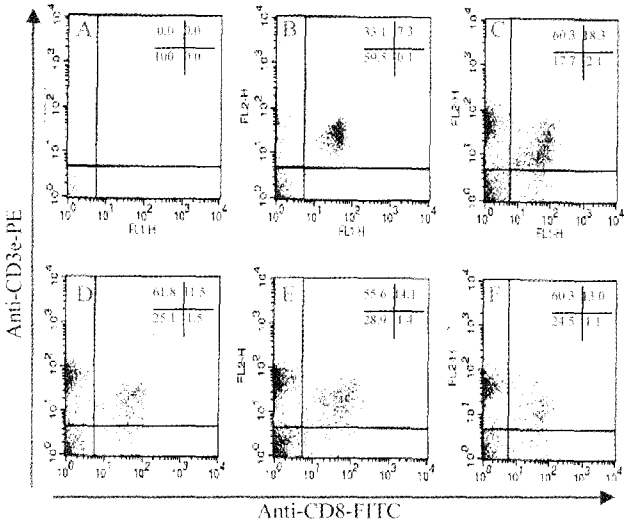

Fig. 5. Effects of fungal extract on proliferation of $CD3e^+$ and $CD8^+$ in PBMCs of healthy murine. The PBMCs were isolated from Balb/c mice and cultured in the absence or presence of a fungal extract (each 100 μg/ml) for 72 h and analyzed using a flow cytometer. The events in panel (A) were backscattered onto a dot-plot of SSC versus FSC and CD3e-PE versus CD8-FITC (B to F). B, no treatment; C, concanavalin A; D, *C. sinensis*; E, *B. bassiana*; F, *C. hepialidicola*.

respectively. The subpopulation of $CD3e^+/CD8^+$ was also analyzed by flow cytometry (Fig. 5). The $CD3e^+/CD8^+$ frequencies of PBMC cultured with fungal extracts were 11.5 (*C. sinensis*), 14. 1 (*B. bassiana*), and 13.0% (*C. hepialidicola*). Compared to the subpopulation of the untreated control, the frequency of $CD3e^+/CD8^+$ as well as $CD3e^+/CD4^+$ increased significantly. This is likely due to the pro-

liferation of both subsets. The $CD4^+/CD8^+$ T cell ratio of untreated cells is 4 : 1 and the ratio of cells stimulated with fungal extracts is also approximately similar to that of untreated cells. This means that the $CD4^+/CD8^+$ T cell ratio is not changed in PBMC cultured with fungal extracts. Together with the results of enhanced transcription of cytokines, these results suggest that certain factors in the fungal extracts induced the proliferation of T cells as a mitogen and/or the proliferation and activation of T cells via cytokine production as a polyclonal activator.

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