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Effect of a hot water extract of Agaricus blazei fruiting bodies(CJ-01) on the intracellular cytokines level in a patient with bronchitis

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Abstract

Effect of an oral intake of a hot water extract of Agaricus blazei (CJ-01) on the levels of intracellular cytokines was examined in a bronchitic patient with asthma like attacks. The patient showed an improvement of the symptoms such as frequent cough and sputum after 2 months oral intake of CJ-01. We measured the intracellular production of IL-2 and interferon gamma (IFN-γ), pro-inflammatory Th1 cytokines, as well as IL-10, anti-inflammatory Th2 cytokine, in mitogen-stimulated peripheral blood lymphocytes. Flow cytometric analysis showed an elevation of intracellular IFN-γ level in the lymphocytes of the patient. A deterioration of the symptoms of the patient was observed 3 months after discontinuance of CJ-01. A reduction of IFN-γ level was also observed in parallel. However, resuming oral intake of CJ-01 showed again an ameliolation of the symptoms and the elevation of IFN-γ level was repeatedly observed. The patient further acquired a resistance to catching colds.

These results suggest that the immune enhancing effect of CJ-01 may improve the symptoms of bronchitis through a promotion of pro-inflammatory cytokine production.

Key words Agaricus blazei (CJ-01), bronchitis, flow cytometry, intracellular cytokines, interferon gamma, Th1 and Th2.

Introduction

Antitumor effects[1-3] or immunostimulatory effects[2,4,5] of Agaricus blazei are recently reported. However, less is known about the relation between Agaricus blazei and respiratory diseases. Further, no uniformity has been observed with its medicinal effect and thus there are some problems in these materials from clinical aspects. Recently, we established a culturing method for harvesting fruit bodies with stable constituents by use of the best cytogenetical technique for Agaricus blazei (CJ-01)[6] which can contribute to the use of immunological and pharmacological approaches.

Our previous study showed that a hot water extract of Agaricus blazei (CJ-01) significantly suppressed the blood pressure in a dose-dependent manner in hypertensive SHR rats.[7] Based on this animal study, we examined oral intake of CJ-01 on a volunteer patient with hypertension and bronchitis.

The improvement of respiratory symptoms was observed in this patient, even though hypotensive effect was minimum. Then, we further investigated the immunomodulating effect of CJ-01.

Helper T cell type 1 (Th1) mediate cellular immunity and inflammatory responses, and Th2 cells support humoral responses.[8] An imbalance between Th1 and Th2-associated cytokines appears to be actively involved in the pathogenesis and progression of many diseases such as, some autoimmune diseases,[9-11] cancer[12-14] and

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HIV infection.[15,16] However, few reports have been found about the relationship between Th1/Th2 imbalance and respiratory diseases.

In this study, the immunomodulatory effect of CJ-01 was examined using a novel, highly efficient multiparameter flow cytometric assay that detects the rapid intracellular accumulation of cytokines after short-term (4 h) *in vitro* antigen stimulation. This assay allows the simultaneous assessment of multiple cytokines in single effector T cells.

Subject and Methods

Subject: The patient was a 51-year-old man who had a weak constitution since his childhood and often caught colds leading easily to bronchitis. He had tuberculotic pleuritis at 5 years of age, a repeated duodenal ulcer in his late 30's and cerebral hemorrhage at 46 years of age. Cerebral hemorrhage was induced by multiple factors, such as hypertension, excessive stress and severe frequent coughing after bronchitis. After the cerebral hemorrhage, his blood pressure was well controlled by a low dose of calcium antagonist. He had often been suffering from acute or chronic bronchitis with asthma like attacks since his late 30's. His asthma like attack showed wheezing, dyspnea, orthopnea and frequent sputum expectoration. The treatment for his respiratory symptoms was principally based on the administration of Kampo medicines like Xiao-Qing-Long-Tang (小青竜湯), Qing-Fei-Tang (清肺湯) or Zhu-Ru-Wen-Dan-Tang (竹茹温胆湯) with occasional use of additional antibiotics.

On April 6, 1999, he had a severe asthma like attack with wheezing, frequent sputum expectorations and dyspnea at midnight and visited the Department of Respiratory Medicine of Yamaguchi University Hospital the same morning. He was treated as a patient with bronchial asthma by inhalations of bronchodilator and steroid and a drip infusion of aminophyllin. After one week of treatment with western medicines, his symptoms were stabilized and he wanted to start taking Kampo medicine. Xiao-Qing-Long-Tang was prescribed.

His initial laboratory data is shown in Table I.

Physical examination revealed a height of 171 cm and a weight of 52 kg. His body temperature was 36.5 ℃, pulse was 96/min and regular, and blood pressure was 156/109 mmHg.

On auscultation, wheezing and piping sounds were heard from his chest but the heart sound was normal. There was no edema or eruption of the skin, and the conjunctivas were not anemic or icteric, and tonsils and thyroid were not swollen. The liver, spleen and kidneys were not palpable. There was no neurological abnormality except numbness of left leg due to the sequela of old cerebral hemorrhage.

In a chest X-ray, CTR was 37% and an adhesive dull edge of left costo-phrenic angle was observed, suggesting a scar of old pleuritis. Electro-cardiogram was normal except tachycardia (H.R. : 100/min).

As shown in Table I, although some abnormal data were observed, they were usually normal or controlled well. Then, they were considered as a transient response according to the acute respiratory episode at that time. His C-reactive protein (CRP) was positive (1.17) , blood sugar (BS) was 172, and blood pressure (BP) showed 156/109. One month later, they were normalized on May 11, 1999. (CRP 0.1, BS 81, BP 136/88)

After the respiratory episode of April, 1999, he begun to take CJ-01 voluntarily. However, the initial aim of taking CJ-01 was for his hypertension.

The patient took a hot water decoction of CJ-01 (5 g/day) for 2 months and blood pressure was monitored every morning. Intracellular production of cytokines (IL-2, IFN-γ and IL-10) were measured by flow cytometry before and after taking CJ-01. The clinical status about respiratory symptoms was also monitored.

Flow cytometric analysis of intracellular cytokines: Flow cytometric determination of IFN-γ and IL-2 in the cytoplasm of peripheral lymphocytes was performed by a previously described method.[17] Briefly, to determine the potential for Th1-associated cytokine production (IFN-γ and IL-2), aliquots (500 μl) of heparinized whole blood obtained from a patient was stimulated with a combination of 25 ng/ml of phorbol myristate acetate (PMA; Sigma, St. Louis, MO) and 1 μg/ml of ionomycin in the presence of 10 μg/ml of brefeldin A (BFA; Sigma, St. Louis, MO) and cultured for 4 hours at 37℃ in a humidified incubator containing 5% CO_2. Brefeldin A (a relatively non-toxic, but potent, inhibitor of intracellular transport that prevents secretion of any produced cytokines) was used to increase the sensitivity of cytokine detection.

For Th2-associated cytokine analysis (IL-10), lymphocytes were stimulated with 5 μg/ml of Concanavalin A (ConA; Sigma, St. Louis, MO) in the presence of brefeldin and cultured for 4 hrs.

After staining T lymphocytes with anti-CD3-PerCP monoclonal antibody (mAb) (Becton Dickinson, San Jose, CA), the permiabilizing solution (Becton Dickinson, San Jose, CA) to permiabilize the lymphocyte membrane was added. Finally, the samples were incubated with anti-human IL-2-PE (Becton Dickinson, San Jose, CA), IFN-γ -FITC (Th1) (Becton Dickinson, San Jose, CA), and IL-10-PE (Th2) mAbs (Caltag lab., Burmingham, CA) for 30 min to bind each cytokine produced during activation and retained intracellulary. After fixation, flow cytometric analysis was carried out by FACScan (Becton Dickinson, San Jose, CA). For each analysis, after counting at least 10,000 lymphocytes gated by positive CD3 expression, the percentage of IFN-γ and IL-2-positive cells (for Th1) and IL-10 positive cells (for Th2) was counted by FACScan.

Results

Time course of Th1- and Th2-associated cytokines

When the patient had a severe asthma like attack in the beginning of April, IFN-γ level was 41.9% and IL-10 level was high and its positivity was 5.2%. After taking CJ-01 for 2.5 months, IFN-γ level elevated to 71.7-61.4% and IL-10 level was reduced to 0.2% (Figure 1). The result of flow cytometric analysis of IFN-γ and IL-2 dated on July 30 is shown in Figure 2a and 2b. IFN-γ positive cells exist in the right upper and lower quadrants and IL-2 positive cells exist in the upper right and left quadrants in Figure 2b.

The patient showed an improvement of respiratory symptoms, such as cough and sputum with sleep disturbance in the night. The patient had to wake up frequently to expectorate the sputum which accumulated while he was asleep. However, the frequency of sputum expectoration decreased from 5-7 times to once or less per night. In proportion with the improvement of symptoms, the elevation of IFN-γ and the reduction of IL-10 were observed. Discontinuance of taking CJ-01 for 3 months caused a deterioration of the respiratory symptoms including asthma like attack and the reduction of IFN-γ (16.2%) was observed (Figure 1).

Since the patient begun to expectorate sputum frequently in the night, he resumed taking CJ-01 and the symptoms were ameliolated. The elevation of IFN-γ level was repeatedly observed. The patient further acquired a resistance to catching colds.

Half a year later, the patient discontinued CJ-01 intake again and his respiratory symptoms deteriorated. Then, he resumed CJ-01 intake and intracellular cytokine levels were monitored again for two weeks. Before taking CJ-01, the patient complained of 6-7 expectorations of sputum in the night and IFN-γ level was 27.5%. After taking CJ-01, the increase in IFN-γ level and the decrease in IL-10 level were observed (Table II) and the respiratory symptoms were ameliolated (1-2 expectorations of sputum in the night). Further, even after discontinuing to take CJ-01 completely, the patient had acquired a resistance to catching colds for two years. However, in the second year without CJ-01, the respiratory symptoms like cough, frequent sputum expectorations in the night and asthma like attacks gradually increased

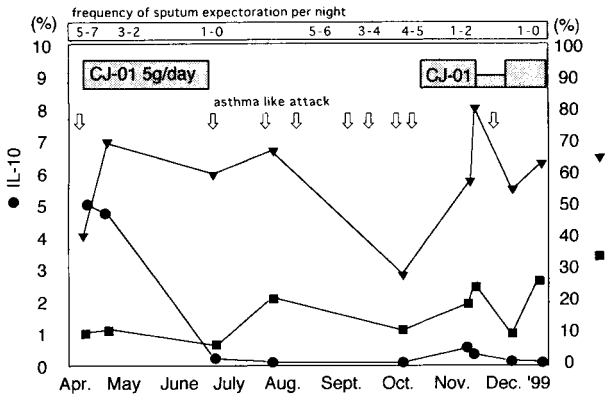

Fig. 1 Time course of intracellular cytokines level and respiratory symptoms

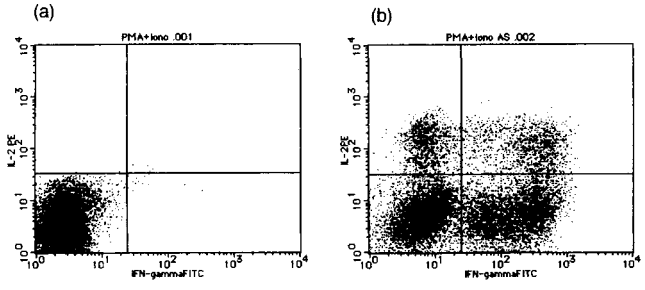

Fig. 2 Distribution of intracellular interferon gamma and IL-2. Interferon gamma-and IL-2-positive cells are distributed in the right two quadrants and upper two quadrants respectively in the presence of antibodies (b), and no positive cell is observed in the absence of antibodies (a).

Table I. Results of hematological and chemical laboratory data

RBC 469, Ht 44.4, Hb 15.3, WBC 4100(Eosin 2.5), Plt 12.4
TP 6.5, Alb. 3.4, CRP 1.17, TB 0.6, DB 0.2, BS 172, ChE 168, Tchol 191, UN 16, Crea 0.67, GOT 24, GPT 25, LDH 185, AlP 208, γ -GTP 15, IgE 39, Na 141, K 4.0, Cl 104

Table II. Time course of IFN- γ level after resuming to take CJ-01 again.

		day 0	day 1	day 3	day 7	day14
	date	000516	000517	000519	000523	000530
Th 1	IFN- γ	27.5	36.9	51.2	65.3	56.9
	IL-2	69.1	28.4	31.5	31.1	16.2
Th 2	IL-10	8.1	0.7	1.2	3.4	1.5

and the levels of intracellular IFN- γ and IL-10 were 29.3 % and 4.0 % respectively on May 15, 2002.

Discussions and Conclusions

Several reports concerning antitumor effects or immunostimulatory effects of Agaricus blazei have been recently presented.[1-5] Further, our previous animal study showed that a hot water extract of Agaricus blazei (CJ-01) reduced the blood pressure of spontaneous hypertensive rats *in vivo*.[7] Based on this result, a patient voluntarily took CJ-01 as a functional food under an agreement to participate in the study with informed consent. Although the effect of CJ-01 on the blood pressure was minimum (data not shown), the patient showed an improvement of symptoms, such as frequent cough and sputum with sleep disturbance in the night. The improvement of symptoms was accompanied by the elevation of IFN- γ production in lymphocytes from the patient. Figure 1 clearly showed a parallel correlation between the improvement of clinical symptoms and the elevation of pro-inflammatory Th1 cytokine production. Table I also showed that the elevation of IFN- γ level was observed in one to two weeks and the improvement of respiratory symptoms was accompanied.

Less is known about the relation between respiratory diseases and Agaricus blazei as well as Th1/Th2 imbalance and respiratory diseases, though Bont mentioned about the disease severity in respiratory syncytial virus (RSV) bronchiolitis and peripheral blood cytokine responses.[18] Our study revealed that CJ-01 improved the symptoms of the patient and elevated IFN- γ production at the same time.

Renzi *et al.*[19] reported that lower IFN-gamma production at the time of bronchiolitis is an indicator of lower pulmonary function and increased responsiveness to histamine 4.9 mo after bronchiolitis and is related to the development of asthma after bronchiolitis in infants. In this study, when the patient showed increased asthma like attacks or frequent sputum in the night, IFN- γ level was relatively low and IL-10 level was high.

IL-2 and IFN- γ are known as pro-inflammatory Th1 cytokines mediating cellular immunity and IL-10 as anti-inflammatory Th2 cytokine supporting humoral responses.[8] Animal study by Wissing *et al.*[20] showed that pre-treatment of an anti-CD3 monoclonal antibody caused a profound deficit in both IL-2 and IFN- γ secretion upon restimulation *in vivo*, whereas IL-10 production was significantly increased. Ito *et al.*[21] reported that IL-10 inhibits expression of both interferon alpha- and interferon gamma-induced genes by suppressing tyrosine phosphorylation of STAT1. Therefore, suppression of IL-10 production or promotion of IL-2 and IFN- γ production may restore Th1/Th2 imbalance and also contribute to improve respiratory symptoms or prevent the development of pulmonary diseases. In this study, the patient acquired a resistance to catching colds, suggesting that CJ-01 may have an immune enhancing effect through a promotion of pro-inflammatory cytokine production.

Sakaguchi *et al.*[22] reported that the IFN- γ level of the group with liver cirrhosis (LC) without hepatocellular calcinoma (HCC) was 29.7 $\pm$ 2.8 % and significantly lower than that of the groups with LC and HCC (44.2 $\pm$ 3.7 %) or age/gender-matched controls (49.5 $\pm$ 4.7 %). Tanaka *et al.*[23] reported that the positivity of IFN- γ producing CD4+ cells was 22.6 $\pm$ 8.5 in healthy adults of age 50 - 72. As compared with these data, the positivity of Th1 of the patient in this study was much higher after he took CJ-01 and showed the improvement of respiratory symptoms. In the case of this patient, up-regulation of Th1-associated IFN- γ and down-regulation or maintenance of low level of IL-10 is likely to be required to keep his health status.

In conclusion, we have demonstrated that CJ-01 showed an ameliolation of respiratory symptoms followed by an elevation of Th1-associated IFN- γ and reduction of Th2-associated IL-10. These findings suggest that CJ-01 may be a potent therapeutic medicinal herbal

extract in respiratory diseases. Further study should be required to elucidate the effieacy of CJ-01 in immunomodulation.

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和文抄録

喘息様発作を伴う気管支炎患者において，アガリクス熱水抽出物（CJ-01）服用前後で末梢血リンパ球における細胞内サイトカインレベルを Th1 系では IL-2 および IFN-γを，また Th2 系では IL-10 を測定した。CJ-01 を 2 ヶ月服用した際には，頻回の咳，痰などの症状改善がみられ，また Flow Cytometry 解析では CJ-01 服用後に IFN-γの上昇がみられた。CJ-01 服用中止後 3 ヶ月で呼吸器症状の悪化がみられ，これと平行して IFN-γレベルの低下が認められた。しかしながら CJ-01 服用再開後には，症状の改善と IFN-γレベルの上昇が認められた。患者にはさらに風邪をひきにくくなるなどの自覚もあった。これらの結果は CJ-01 の免疫増強効果が IFN-γ産生増強を介して気管支炎症状を改善したことを示唆するものである。

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