BPB Reports

Report

Immunomodulatory Activity of an Aqueous Extract of Mycelium of an Endophytic-Like Fungus, *Ustilago esculenta*

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Received March 8, 2021; Accepted June 30, 2021

An aqueous extract of mycelium of *Ustilago esculenta,* an endophytic-like fungus, was intraperitoneally administered to mice with a model antigen (NP-BSA) and aluminum adjuvant (Alum). Blood was periodically collected from the tail veins of the mice, and the antibody titer against NP-BSA and/or BSA in the serum was measured by ELISA. The aqueous *U. esculenta* extract had no significant effect on the IgM and IgG antibody titers in the primary response but greatly lowered those in the secondary response. When the antibody titers of the IgG subtypes were separately measured, the ratios of IgG2b/IgG1 and IgG2c/IgG1 titers in the mice treated with the *U. esculenta* extract were markedly lower than those in the control mice treated with NP-BSA/Alum. The Bio-Plex™ cytokine assay revealed that the concentrations of interferon-γ (IFN-γ) and interleukin-12 (IL-12 (p70)) in peripheral blood from *U. esculenta* extract-treated mice were decreased as compared with those from the control mice three weeks after the initial immunization. These results suggest that the aqueous extract of mycelium of *U. esculenta* suppressed antibody production in the secondary response and that changes of the Th1/Th2 balance were involved in this process.

Key words immunomodulation, *Ustilago esculenta*, smut fungus, IgG subtype, Th1/Th2 balance

INTRODUCTION

Ustilago esculenta is an endophytic-like fungus belonging to the Ustilaginaceae, a family of smut fungi. Colonization of the aquatic wild perennial plant *Zizania lantifolia* by *U. esculenta* specifically leads to the formation of a swollen structure called the smut-gall in the flowering stem, thereby interfering with inflorescence and seed formation. The smut-gall harboring the fungus has been eaten by people in Asian countries for more than 400 years; the edible gall is commonly referred to as "ishing-kambong" in India,[1] "jiaobai" in China, "gausun" in Taiwan,[2,3] and "makomotake" in Japan.[4] The interaction between *Z. latifolia* and *U. esculenta* has been extensively studied to uncover the mechanism of their endosymbiosis.[5] The mycelia of *U. esculenta* and *Z. lantifolia* infected with *U. esculenta* have also been utilized as folk medicines since ancient times. However, a systematic pharmacological study has not been conducted, and thus knowledge of the medicinal efficacy of the mycelia is limited. Nagasaka *et al*. investigated pharmacological actions of an aqueous extract of *U. esculenta* on the digestive system of guinea pigs and found that the extract induced acetylcholine-like reactions on isolated ileal smooth muscles.[6] Kawagishi *et al*. then examined the effects of steroid-like compounds isolated from infected smut-gall on bone metabolism in mice and revealed that the compounds suppressed the formation of osteoclasts.[4] In recent years, numerous researchers have focused on the immunostimulating activity of components derived from Eumycetes, including mushrooms and fungi (reviewed in 7–9). It has also been reported that *U. esculenta*-infected *Z. lantifolia* stimulated the production of β-defensin, which plays a crucial role in innate immunity.[10] In this study, we examined the pharmacological effects of *U. esculenta* extract on the humoral immune responses in mice.

MATERIALS AND METHODS

Reagents and Antibodies Aluminum hydroxide adjuvant (Alum) and *p*-nitrophenylphosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) conjugated with (4-hydroxy-3-nitrophenyl)acetyl (NP-BSA; approximately 13 nitrophenyl residues per BSA molecule) was a product of Biosearch Technologies (Petaluma, CA, USA). Alkaline phosphatase (AP)-conjugated goat anti-mouse IgM and anti-mouse IgG were purchased from Southern Biotech (Birmingham, AL, USA).

Preparation of an Aqueous Extract of *Ustilago esculenta* Mycelium The freeze-dried mycelium of *U. esculenta* was a gift from Dr. Akira Hara (Faculty of Agriculture, Meijo University, Nagoya, Japan).[11] The mycelium (0.5 g) was suspended in phosphate-buffered saline (PBS) (5 mL), and the suspension was vigorously shaken by a vortex mixer for 5 min and allowed to stand at 4ºC for 3 d. The suspension was then centrifuged at 15,000 rpm and 4ºC for 5 min, and

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the supernatant was obtained and used as a crude PBS extract of *U. esculenta* mycelium. We further treated the crude PBS extract by chloroform/methanol (C/M) extraction. To the crude PBS extract (2.8 mL), chloroform (0.6 mL) and methanol (0.6 mL) were added, and the solution was mixed by vigorous shaking. After being allowed to stand for 5 min, the mixture was centrifuged at 3,000 rpm for 3 min at 4ºC, and the upper aqueous layer was recovered. To the upper layer, 1-butanol (0.6 mL) was added, and the mixture was vigorously shaken. After standing for 5 min, the mixture was centrifuged at 3,000 rpm for 3 min at 4ºC. The lower layer was used as an aqueous extract for immunization. The chemical analysis of the aqueous extract showed that it contained proteins (0.95 mg/mL) and carbohydrates (1.84 mg/mL) as determined by the bicinchoninic acid (BCA) method and phenol-sulfuric acid method, respectively.

Immunization of Mice Specific pathogen-free mice (C57BL/6N, female, 6-weeks old) were supplied by Sankyo Lab Service Corp. (Tokyo, Japan). A model antigen (NP-BSA) (100 μg/mouse) was intraperitoneally (i.p.) administered to mice with or without aluminum hydroxide adjuvant (Alum) (500 μg/mouse) and an aqueous extract of *U. esculenta* mycelium (100 μL/mouse) as the initial immunization. On Day 49, the mice were injected i.p. with NP-BSA (100 μg/mouse) alone to examine the secondary immune response. There was no effect on the appearance and survival rate of the treated mice. All procedures for experiments using mice were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Hoshi University School of Pharmacy and Pharmaceutical Sciences. The animal protocols were approved by the Animal Care and Use Committee of Hoshi University School of Pharmacy and Pharmaceutical Sciences (protocol #09-78). All experiments were performed under anesthesia and were designed to minimize suffering.

Antibody Determination by ELISA NP-BSA (0.2 mg/mL, 50 μL) was absorbed on ELISA plates (Costar #9031) at 4ºC. The plates were then blocked with 2% BSA/PBS (100 μL) for 1 h and washed with wash buffer (0.05% Tween-20/PBS) three times. Diluted serum samples (1:100) were placed in the wells, and the plates were incubated for 2 h. After unbound materials were removed by washing three times with wash buffer (250 μL), AP-conjugated goat anti-mouse IgM or anti-mouse IgG (1:2000 dilution in PBS, 50 μL) was added and the samples were incubated for 1 h. The wells were washed with wash buffer (250 μL) three times, and the colorimetric assays were performed by incubation with *p*-nitrophenylphosphate (1 mg/mL, 50 μL) for 1 h followed by measurement of absorbance at 415 nm. The collected sera were diluted with PBS for 1:25, 1:50, 1:100, 1:200. The measured value was corrected based on the standard serum. In order to measure the time-course change, the dilution condition in which the ELISA measurement value was not saturated was 1:100 in all the samples. These results are shown in figures.

Bio-Plex™ Cytokine Assay Cytokines in mouse serum were determined by using a Bio-Plex™ suspension array system (Bio-Rad Laboratories, Hercules, CA, USA).[12] Interleukin (IL)-2, IL-4, IL-5, IL-10, IL-12, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and granulocyte/macrophage colony-stimulating factor (GM-CSF) were measured according to the manufacturer's protocols.

RESULTS

Effects of an Aqueous Extract of *U. esculenta* Mycelium on Antibody Production Mice were treated with NP-BSA as a model antigen with or without Alum adjuvant and a crude *U. esculenta* PBS extract by i.p. injection, and challenged with NP-BSA alone on Day 49. The serum was prepared after collection of blood from the tail veins of the mice on Days 7 and 56, and the antibody titers (IgM and IgG) were measured by ELISA. The IgM titers on Day 7 of sera from mice that had been treated with NP-BSA, NP-BSA/Alum, NP-BSA/*U. esculenta* extract, or NP-BSA/Alum/*U. esculenta* extract showed no significant difference among the experimental groups (Fig. 1 *upper*). On the other hand, the administration of the *U. esculenta* extracts greatly suppressed the IgG titer on Day 56 in mice treated with NP-BSA/Alum, in which the IgG titer was greatly elevated after repeated immunizations (Fig. 1, *lower*). These results suggest that the PBS extract of *U. esculenta* has a potent suppressive effect on the secondary IgG response of antibody production induced by the model antigen.

Time-Course Experiments and Analysis of Antibody Subtypes We next conducted more detailed time-course experiments by using C/M-treated PBS extract of *U. esculenta*. When mice were administered the *U. esculenta* extract with NP-BSA/Alum, the primary responses of both IgM and IgG until Day 21 were shown to be slightly increased (Fig. 2, *left*); however, the differences were not statistically significant. By contrast, the IgM responses induced by NP-BSA/Alum on Days 56 and 63 were suppressed by the *U. esculenta* extract (Fig. 2, *right*). Furthermore, the IgG secondary responses on Days 63 and 70 were more effectively suppressed by the administration of the extract. It was thought that the *U. esculenta* extract showed potent suppressive effects on IgG production, we examined whether the extract has differential effects on the production of IgG subtypes. The titers of IgG1, IgG2b, and IgG2c were separately determined by ELISA, and the ratios of IgG2b/IgG1 and IgG2c/IgG1 were calculated (Fig. 3). The results showed that the administration of the *U. esculenta* extracts decreased both the IgG2b/IgG1 and IgG2c/IgG1 ratios, suggesting that the extract indeed has differential effects on the production of IgG subtypes and induces selective suppression of the production of IgG2b and IgG2c.

Cytokine Assay Since the *U. esculenta* extract selectively suppressed the production of IgG2b and IgG2c, we analyzed blood cytokine levels in mice administered NP-BSA/Alum with or without *U. esculenta* extract by the Bio-Plex™ suspension array system. The cytokine levels of IL-2, IL-4, IL-5, IL-10, TNF-α, and GM-CSF in the blood samples collected on Days 14 and 21 were not significantly different between the mice treated with and those treated without the *U. esculenta* extract (data not shown). By contrast, the levels of IFN-γ and IL-12 on Day 21 in mice treated with NP-BSA/Alum/*U. esculenta* extract were found to be lower than those in NP-BSA/Alum-treated mice (Fig. 4).

DISCUSSION

In this study, we examined the effects of the aqueous extract (Fig. 1) and the C/M-treated PBS extract (Fig. 2) of *U. esculenta* on antibody production in mice after the administration of a model antigen (NP-BSA) with Alum adjuvant and found that the extract had potent suppressive effects, especial-

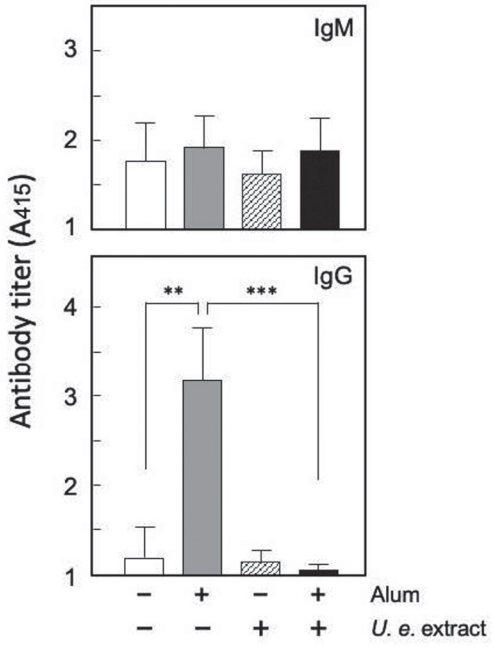

Fig. 1. Effects of an Aqueous Extract of *U. esculenta* Mycelium on Antibody Production

Mice were treated with NP-BSA (100 μg/mouse) in combination with or without Alum adjuvant and a crude *U. esculenta* extract (0.1 mL/mouse) by intraperitoneal (i.p.) injection and challenged with NP-BSA alone (100 μg/mouse) on Day 49. The serum was prepared after collection of blood from the tail veins on Day 7 (for IgM) and Day 56 (for IgG). The antibody titers were measured by ELISA and are expressed as relative values to the control mouse serum. Experiments were performed using four mice in each group, and the data are presented as the mean ± SEM. Statistical data analysis was conducted with t-tests. $^{**}p < 0.01$, $^{***}p < 0.005$.

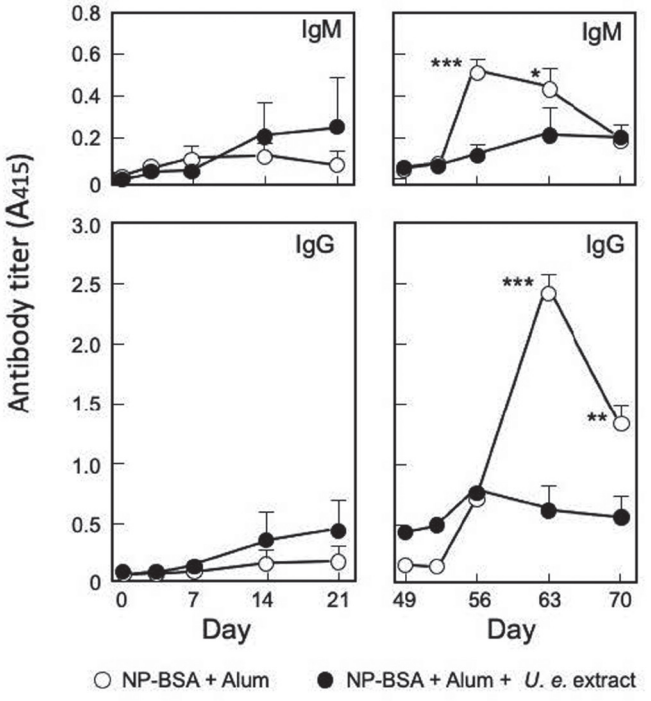

Fig. 2. Effects of an Aqueous Extract of *U. esculenta* Mycelium on the Primary and Secondary Antibody Productions

Mice were intraperitoneally (i.p.) administered NP-BSA (100 μg/mouse) in combination with or without Alum adjuvant and a C/M-treated aqueous extract of *U. esculenta* (0.1 mL/mouse) and challenged with NP-BSA alone (100 μg/mouse) on Day 49. The serum was prepared after periodical collection of blood until Day 70. The antibody titers (IgM and IgG) were measured by ELISA, and A415 values are shown. Experiments were performed using four mice in each group, and the data are presented as the mean ± SEM. Statistical data analysis was conducted with t-tests. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

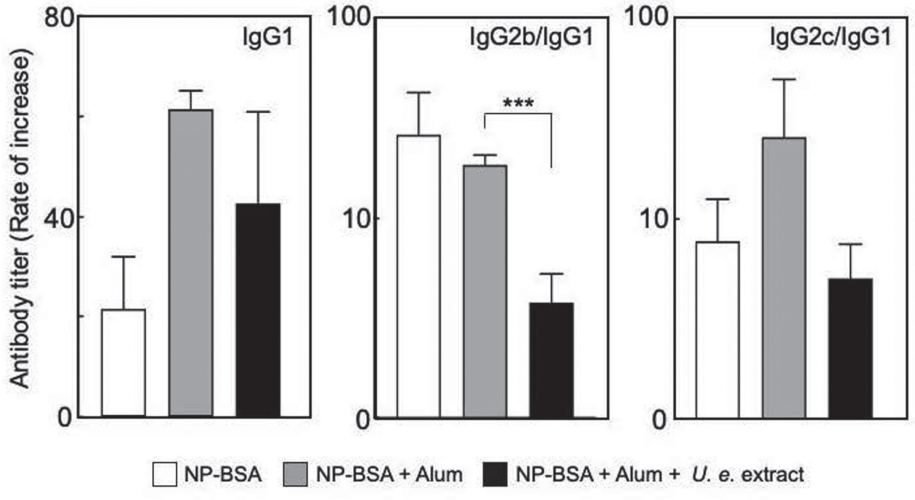

Fig. 3. Differential Effect of an Aqueous Extract of *U. esculenta* Mycelium on the Production of IgG Subtypes

Mice were treated with NP-BSA (100 μg/mouse) in combination with or without Alum adjuvant and an aqueous extract of *U. esculenta* (0.1 mL/mouse) by intraperitoneal (i.p.) injection and challenged with NP-BSA alone (100 μg/mouse) on Day 49. The serum was prepared after collection of blood from the tail vein on Day 56. The antibody titers (IgG1, IgG2b, and IgG2c) were separately measured by ELISA, and the ratios (IgG2b/IgG1 and IgG2c/IgG1) are expressed as relative values to those obtained from NP-BSA-treated mice. Experiments were performed using four mice in each group, and the mean of the ratios are presented. Experiments were performed using four mice in each group, and the data are presented as the mean ± SEM. Statistical data analysis was conducted with t-tests. $^{***}p < 0.005$.

ly on secondary IgM and IgG responses. Since the substances of the extracts differed due to the difference in the extraction methods, it was considered that the degree of the induced IgG antibody titer was different. The chemical analysis of the aqueous extract showed that it contained proteins (0.95 mg/mL) and carbohydrates (1.84 mg/mL). In general, protein antigens elevated IgG with a T cell-dependent response. Therefore, some substances derived from *U. esculenta* might influ-

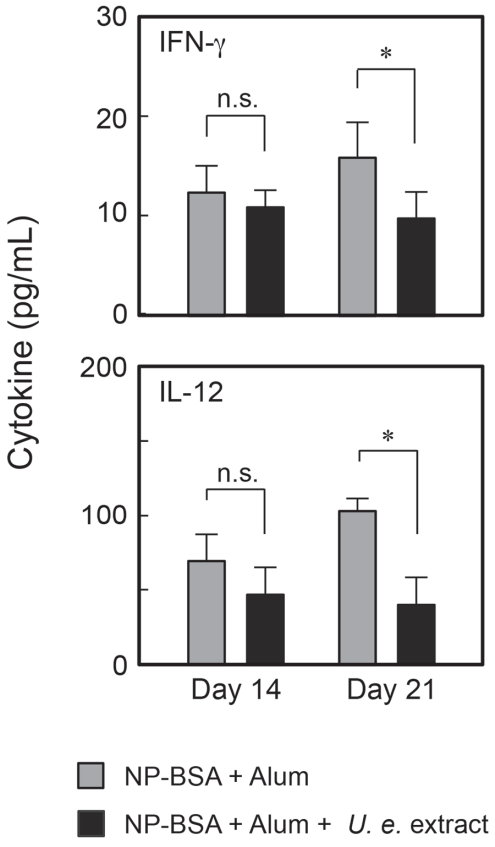

Fig. 4. Measurement of Cytokines by Bio-Plex™ Assay

Mice were treated with NP-BSA (100 μg/mouse) in combination with or without Alum adjuvant and an aqueous extract of *U. esculenta* (0.1 mL/mouse) by intraperitoneal (i.p.) injection. Serum was prepared after collection of blood from the tail veins on Day 14 and 21. The Bio-Plex™ assay was carried out as described previously.[12] Experiments were performed using three mice in each group, and the data are presented as the mean ± SEM. Statistical data analysis was conducted with t-tests. *$p < 0.05$.

ence the response not only as an adjuvant but the regulator of T cells activated by the mixed state of NP-BSA and the substance. It was considered that the high carbohydrate content also influenced that the IgG induction was weakened in the C/M-treated PBS extract.

As the time-course results (Fig. 2), the total IgG antibody titers were suppressed in mice with the C/M-treated extract. In order to investigate the immunological memory status, we focused on the antibody titers on Day 56 (the 7th day of the secondary response), and the results are shown in Fig. 3. There was no significant difference in total IgG antibody titers at this time point (Fig. 2), but when the IgG1, IgG2b, IgG2c subtypes were measured, IgG1 was slightly suppressed by the C/M-treated extract. The ratio of IgG2b/IgG1 and IgG2c/IgG1 was decreased (Fig. 3). Therefore, it was considered that the productions of IgG2b and IgG2c were suppressed. The induction of IgM was also suppressed. There are several possible effects, but we concluded that the C/M-treated extract particularly suppresses the production of IgG2b and IgG2c by Alum adjuvant.

The extract appeared to selectively suppress the production of IgG2b and IgG2c, suggesting that the *U. esculenta* extract shifts the balance of helper T cell subsets to Th2-dominant immunity. It has been reported that the class switch of IgG subclasses is regulated by the Th1/Th2 balance.[13,14] Because we found lowered expression levels of IFN-γ and IL-12 (Th1-inducing cytokines) on Day 21 in mice treated with the *U. esculenta* extract, the extract might affect the class switch of IgG through decreased induction of Th1 cells.

Over the three weeks after the initial immunization, we found a slight increase in the antibody responses, and the formation of memory cells was likely induced in this period. Because the aqueous *U. esculenta* extract had inhibitory effects on the secondary responses to the treatment with antigen alone, it was strongly suggested that the extract had suppressive effects on the induction of Th1-type memory and the secondary antibody responses. In the future, more detailed analyses will be needed to provide mechanistic insights into the suppression.

Nagasaka *et al*. suggested that an aqueous extract of *U. esculenta* induces acetylcholine-like reactions on isolated ileal smooth muscles.[6] It is known that T and B lymphocytes express both muscarinic and nicotinic acetylcholine receptors.[15] The relationship between the immunomodulating activity suggested in the present study and the acetylcholine-like activity should be an important issue to be clarified in future studies. Identification of the active ingredients in the *U. esculenta* extract is another important subject. A variety of polysaccharides from fungi and mushrooms have been reported to possess immunomodulating activity.[7–9] Because the extract contains considerable amounts of carbohydrates, polysaccharides or related glycoconjugates might be active substances.

It has previously been shown that selective production of IgG subclasses is involved in the pathogenesis of certain autoimmune diseases, e.g., the increased levels of Th2-driven IgG subclasses, IgG2b and IgG2c, are correlated with exacerbation of experimental autoimmune myasthenia gravis (EAMG).[16] Some components derived from the *U. esculenta* extracts will hopefully improve the autoimmune disease. It has also been reported that the IgG2c subclass is important for the immune response to β-amyloid (Ab) in amyloid precursor protein/transgenic mice.[17] The present study may provide valuable information on the pathogenesis of and immunotherapy for neurodegenerative diseases such as Alzheimer's disease.

Acknowledgments We would like to thank Dr. Akira Hara (Meijo University, Japan) for the kind gift of the freeze-dried mycelium of *U. esculenta*. We are also grateful to Dr. Yusuke Ando and Ms. Kotomi Kawai (Hoshi University School of Pharmacy and Pharmaceutical Sciences) for their technical assistance and helpful discussion. This work was supported in part by a scholarship donation to Hoshi University from Dr. Junko Nagasaka, and by a Grant-in-Aid for Scientific Research and a grant from the Program for the Strategic Research Foundation at Private Universities, the Ministry of Education, Culture, Sports, Science and Technology of Japan (#22590115 and #18K07334).

Conflict of interest The authors declare no conflict of interest.

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