Secretion of TNF-α, IL-8 and Nitric Oxide by Macrophages Activated with *Agaricus blazei* Murill Fractions in Vitro

Kenji Sorimachi1*, Kazumi Akimoto2, Yukari Ikehara3, Keiichiro Inafuku3, Akira Okubo4, and Sunao Yamazaki4

1Department of Microbiology and 2Institute of Medical Research, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293, Japan, 3Okinawa Fermentative Chemicals Co. Ltd., Nishizakicho, Itoman, Okinawa 901-0305, Japan, and 4Department of Applied Biological Chemistry, The University of Tokyo, Yayoi, Bunkyo, Tokyo 113-8657, Japan

**ABSTRACT.** Water extracts of the mycelial culture and fruiting bodies of *Agaricus blazei* Murill were fractionated by ethanol precipitation using various ethanol concentrations. Original water extracts from mycelia (Fraction A-0) and fruiting bodies (Fraction B-0) induced TNF-α secretion by macrophages derived from rat bone marrow. Fractions B-4 and B-5 obtained from ethanol precipitation of fruiting bodies using 44% and 50% ethanol, respectively, and Fraction B-6 obtained from the supernatant at 50% ethanol markedly induced TNF-α secretion. Similar effects were observed in IL-8 secretion by macrophages. Regarding nitric oxide (NO), Fraction B-5 induced a significant increase in NO secretion and Fractions B-4 and B-6 induced slightly NO secretion. Northern blot analysis showed that the increases in cytokine- and NO secretion were due to an increase in cytokine mRNAs or NO synthase mRNA. Therefore, it is concluded that *Agaricus blazei* Murill components which activate macrophages result in the induction of cytokine- and NO secretion *in vitro*.

**Key words:** *Agaricus blazei* Murill/macrophages/TNF-α/IL-8/nitric oxide (NO)

Water-solubilized lignin (EP3) extracted from the culture medium of edible Japanese mushroom (*Lentinus edodes*) mycelia shows anti-viral activity *in vitro* (Suzuki et al., 1989b; Sorimachi et al., 1990b). In addition, lignosulfonate (LS) obtained from the waste liquor from the acid sulfite pulping process of wood shows similar effects (Suzuki et al., 1989a, 1989b; Sorimachi et al., 1990a). In our recent study (Sorimachi et al., 2001), *Agaricus* fractions showed anti-viral activity for western equine encephalitis (WEE) virus *in vitro*. We have shown that water-solubilized lignin (EP3) induces the secretion of cytokines such as TNF-α and IL-8, and nitric oxide (NO) by macrophages *in vitro* (Sorimachi et al., 1999). This cytokine secretion was observed by lipopolysaccharide (LPS) or conditioned media of cultured cells (Tsuru et al., 1995). These phenomena may be due to macrophage recognition of stimuli as foreign bodies. In the present study, therefore, *Agaricus* components fractionated with ethanol have been examined to determine whether they induce cytokine- and NO secretion by macrophages *in vitro*.

Lignin derivatives (EP3 and LS) show not only anti-tumor activity (Sorimachi et al., 1997), but also macrophage-activating activity (Sorimachi et al., 1999). Recently, the extracts obtained from *Agaricus blazei* Murill have been used as functional foods, and it apparently seems to be the case that the extracts show anti-tumor activity. However, it is unclear whether the extracts directly attack tumor cells or activate the immune system, resulting in the anti-tumor activity. Therefore, the present study has been designed to determine whether *Agaricus* components activate *in vitro* macrophages which positively contribute to the immune system.

**Materials and Methods**

**Preparation of Agaricus mycelial extract**

*Agaricus blazei* Murill was cultured in a solid medium of sugar
cane bagasse and defatted rice bran (9:1) at 25–27°C for about 4 months. Just before the formation of the fruiting bodies, the whole medium was suspended in 4 volumes of hot water (50°C) for one hour to autolyze the mycelia. Autolyzate was added to the hot water and extracted twice. The extract was filtered with celite and spray-dried. The sieved powder is called *Agaricus* mycelial extract, Fraction A-0 (Table I).

**Preparation of Agaricus fruiting body extract**

Dried fruiting bodies of *Agaricus* were pulverized and treated with hot water twice. The residue was further treated with 40% ethanol overnight. Both extracts were combined, filtered and concentrated under reduced pressure. The concentrate was spray-dried to give the fruiting body extracts, Fraction B-0 (Table I).

**Fractionation of Agaricus mycelial extracts and fruiting body extracts**

To the powder of the mycelial extracts, 10 volumes of water (w/v) were added. After the undissolved matter was removed by centrifugation, 1/5 (v/v) of cold ethanol was added and centrifuged at 10,000xg for 20 min at 4°C. The first fraction of 17% ethanol precipitate was designated Fraction A-1 (Table I). The same volume of ethanol was added to the supernatant and centrifuged to make Fraction A-2 (17% ethanol soluble-29% ethanol insoluble fraction). Stepwise, the addition of ethanol up to 50% and the separation of precipitates gave 6 fractions, A-1 to A-6, in which A-6 was the 50% ethanol soluble fraction. To the fruiting body extract, the same ethanol fractionation was carried out and 6 fractions, B-1 to B-6, were obtained (Table I).

**Cytokine and NO assays**

Macrophages derived from rat bone marrow were prepared according to the method reported previously (Saotome et al., 1987; Sorimachi et al., 1990a). Macrophages were cultured in 12-well culture plates, and treated with *Agaricus* fractions for 6 hrs for TNF-α and IL-8 assays, and for 72 hrs for NO assay (Sorimachi et al., 1999). Aliquots (25–50μ) of the culture media were used for their assays. TNF-α and IL-8 assays were carried out with a rat-TNF-α assay kit (Genzyme) and a rat-IL-8 assay kit (Immune Biological Laboratories Co., Fujikawa, Gunma, Japan), respectively. NO was assayed with the Griess reagent kit, purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan).

**Northern blot analysis**

Total RNA was extracted from macrophages cultured in 90-mm culture dishes by the acid guanidium-isoionicyanate phenol method (Chomczynski and Sacchi, 1987). Macrophages were solubilized with Isojen purchased from Wako Pure Chemical Co. Ltd. In order to make the probes for the hybridization, we used RT-PCR. PCR primers synthesized were purchased from Espec Oligo Service (Tsukuba, Japan). The sequences of PCR primers were: TNF-α forward primer, 5'-CAGGGCAATGATCAGTCCAAGTGA-3', reverse primer; 5'-GCAGTCAGATCCTCTGCGA-3' (Gossart et al., 1996), IL-8 (forward primer; 5'-GAAGATAGATTGGCCCGATG-3', reverse primer; 5'-CATACCTCCTACA-CACATTTC-3') (Kamikubo, 1993), NOs (forward primer; 5'-CTGCCAGGTCTTTGACGCTCGG-3', reverse primer; 5'-GTGGGACACAGGGGTGATGCT-3') (Hattori and Gross, 1993).

The Takara RT-PCR assay kit was purchased from Takara. The conditions of RT-PCR were as described in detail in our previous paper (Sorimachi et al., 1999). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified in parallel as a reference using primers that have been described elsewhere (Terada et al., 1992). The products were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide, and were visualized by ultraviolet light. The PCR-products were extracted and purified with the DNA & Concentrator®-100 (Zymo Research, Orange, CA, USA). The labelling of probes was carried out according to manufacturer protocol with the Megaprime DNA labelling system (Amersham Pharmacia Biotech, Buckinghamshire, England).

Electrophoresis of total RNAs were carried out on a 1% agarose gel, and then the RNA was transferred to Nylon membrane (Hybond-N, Amersham Pharmacia Biotech). Hybridization was carried out using PerfectHyb hybridization solution (Toyobo Co. Ltd., Osaka, Japan). The amount of mRNA, detected by hybridization of Northern blots, was quantified by Phosphor-Imager analysis using BAS 2000II (Fuji Film, Tokyo, Japan).

**Results**

**Preparation of Agaricus component solutions**

Each of the dried fractions was dissolved at 1 mg/ml in phosphate buffered saline (PBS), pH 7.4, without Mg²⁺ and Ca²⁺, and sterilized with a membrane filter whose pore size was 0.22 μm. As Fractions B-4, B-5 and B-6 were not dissolved completely in PBS, the solutions were centrifuged at 1,000-xg for 10 min to remove insoluble matters. Regarding Fraction B-5, the small particles were not removed by centrifugation, but they were completely removed by membrane filtration. Therefore, the actual concentrations of Fractions B-4, B-5 and B-6 were below 100 μg/ml at the final stage.

**Table I. Ethanol fractionation of water extracts from *Agaricus mycelia* and fruiting bodies**

<table>
<thead>
<tr>
<th>Ethanol (%)</th>
<th>Fraction (Mycelia)</th>
<th>Yielda (%)</th>
<th>Fraction (Fruiting bodies)</th>
<th>Yielda (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>A-0</td>
<td>100</td>
<td>B-0</td>
<td>100</td>
</tr>
<tr>
<td>17</td>
<td>A-1</td>
<td>8.4</td>
<td>B-1</td>
<td>2.2</td>
</tr>
<tr>
<td>29</td>
<td>A-2</td>
<td>8.6</td>
<td>B-2</td>
<td>2.2</td>
</tr>
<tr>
<td>38</td>
<td>A-3</td>
<td>8.4</td>
<td>B-3</td>
<td>3.4</td>
</tr>
<tr>
<td>44</td>
<td>A-4</td>
<td>5.1</td>
<td>B-4</td>
<td>3.4</td>
</tr>
<tr>
<td>50</td>
<td>A-5</td>
<td>4.9</td>
<td>B-5</td>
<td>2.2</td>
</tr>
<tr>
<td>50 sup.</td>
<td>A-6</td>
<td>69.7</td>
<td>B-6</td>
<td>86.7</td>
</tr>
</tbody>
</table>

*Average of two independent experiments.
Activation of Macrophages with Agaricus Fractions

**TNF-α secretion**

Macrophages treated with various fractions secreted TNF-α as shown in Fig. 1-A. Fractions A-0 and B-0 significantly induced TNF-α secretion by macrophages, and Fractions B-4, B-5 and B-6 markedly induced TNF-α secretion as well (Fig. 1-A). The other fractions slightly induced TNF-α secretion, and these small increases were significant because of an undetectable level of TNF-α secretion in the control cells untreated with Agaricus fractions.

As shown in Fig. 1-B, Fractions A-0 and B-0 showed significant effects on TNF-α secretion at a concentration of 50 μg/ml, whereas no significant effect was observed at concentrations below 10 μg/ml. At a concentration of 100 μg/ml, the effect of Fraction A-0 and B-0 was further increased as compared with that at 50 μg/ml. On the other hand, Fraction B-5 showed a significant effect on TNF-α secretion even at a concentration of 2.5 μg/ml, and the effect increased in a dose-dependent manner between concentrations of 2.5 and 100 μg/ml.

**IL-8 secretion**

Every fraction was incubated with macrophages, and IL-8 secreted by the macrophages was assayed by ELISA (Fig. 2-A). Fractions A-0, B-0, B-4, B-5 and B-6 markedly induced IL-8 secretion, and the magnitude of the effect of Fraction B-4, B-5 and B-6 were almost identical, as shown in Fig. 2-A. Other Fractions slightly induced IL-8 secretion by macrophages. A small secretion of IL-8 was observed in the control cells untreated with Agaricus fractions.

Fraction A-0 showed a significant effect on IL-8 secretion at a concentration of 10 μg/ml and the effect further increased at a concentration of 50 μg/ml (Fig. 2-B). Regarding Fraction B-0, its effect was significant at a concentration of 5 μg/ml and it gradually increased between concentrations of 5 and 50 μg/ml. Fraction B-5 showed a significant effect at 2.5 μg/ml, and the magnitude of the effect was greater than those of Fractions A-0 and B-0 at a concentration of 50 μg/ml. The effect was almost leveled off at 10 μg/ml.

**NO secretion**

When macrophages were cultured in the presence of Agaricus fractions at 100 μg/ml, a great induction of NO secretion was observed with Fraction B-5, and a small induction was observed with Fraction B-4 or B-6, as shown in Fig. 3. No significant effect of the other fractions was observed. To observe a significant effect, the concentration of Fraction B-5 needed to be more than 50 μg/ml (data not shown).

**Northern blot analysis**

To determine whether induction of cytokine secretion and NO secretion is regulated at the transcriptional level in macrophage activation with Agaricus components, the amount of mRNAs of TNF-α, IL-8 and NO synthase (NOS) was assayed by Northern blot analysis.

The band corresponding to TNF-α was observed in the control macrophage untreated with Agaricus fractions, as shown in Fig. 4. Slightly high TNF-α band intensity was seen following induction with Fraction A-0, and high inten-

![Fig. 1. Secretion of TNF-α from macrophages treated with Agaricus fractions. A: macrophages were treated with various Agaricus fractions at 100 μg/ml. B: Dose-response effect of certain Agaricus fractions on TNF-α secretion. The value is the mean ± S.D. for 3 separate experiments.](image)
K. Sorinachi et al.

...intensities of TNF-α/G61 mRNA bands were observed by addition of Fractions B-4, B-5 and B-6. Much higher intensities of IL-8 mRNA bands were observed by addition of Fractions B-4, B-5 and B-6.

In the secretion of NO by macrophages, no significant increase was observed in the macrophages treated with Fraction A-0 or B-0, although Fractions B-4, B-5 and B-6 induced NO secretion. NO secretion was markedly observed by macrophages treated with Fraction B-5 (Fig. 3). No significant change in NOs mRNA band intensity was observed in the macrophages treated with Fractions A-0 or B-0, whereas much high intensities of NOs mRNA bands were observed in the macrophages treated with Fractions B-4, B-5 and B-6, as shown in Fig. 4. The effect of Fraction B-5 was the strongest among these three samples. Therefore, these results are consistent with those obtained from the NO secretion assay.

The intensity of GAPDH, used as a control, was almost constant among the samples (Fig. 4).

Discussion

Fraction B-0 induced TNF-α and IL-8 secretion by macrophages (Figs. 1 and 2), and Fractions B-4, B-5 and B-6 were more effective than Fraction B-0 in inducing cytokine secretion. These results indicate that the components which activate macrophages are concentrated into Fractions B-4, B-5 and B-6 obtained by the precipitation using relatively high ethanol concentrations. On the other hand, Fraction A-0 significantly induced TNF-α and IL-8 secretion by macrophages (Figs. 1 and 2), but the other fractions from A-1 to B-6 were not effective in inducing cytokine secretion.

Fig. 2. Secretion of IL-8 from macrophages treated with Agaricus fractions. A: macrophages were treated with various Agaricus fractions at 100 μg/ml. B: Dose-response effect of certain Agaricus fractions on IL-8 secretion. The value is the mean ± S.D. for 3 separate experiments.

Fig. 3. Secretion of NO from macrophages treated with Agaricus fractions. Macrophages were treated with various Agaricus fractions at 100 μg/ml. The value is the mean ± S.D. for 3–10 separate experiments.
Activation of Macrophages with Agaricus Fractions

A-6, did not induce a higher cytokine secretion than the unfractionated-original Fraction A-0. Thus, the cytokine induction with Fraction A-0 may be due to the synergistic effect of two or more components rather than the loss of effective components during ethanol fractionation.

Regarding NO secretion, Fraction B-5 markedly induced NO secretion by macrophages, and only low level of NO secretion was observed with Fractions B-4 and B-6 (Fig. 3). On the other hand, neither Fractions A-0 or B-0 induced NO secretion (Fig. 3) or NOs mRNA increase (Fig. 4). Thus, the data based on Northern blot analysis are consistent with those based on NO secretion. In our previous studies (Sorimachi et al., 1999), lipopolysaccharide (LPS) induced the secretion of TNF-α, IL-8 and NO as a set of macrophage activation. In the present study, Fractions B-4 and B-6 induced a high level of secretion of TNF-α and IL-8 (Figs. 1 and 2), whereas their effects not only on NO secretion but also NOs mRNA synthesis were smaller than those expected. Thus, the effects of Fraction B-4 and B-6 on TNF-α, IL-8 and NO secretion differed apparently from those of LPS or Fraction B-5. In addition, morphological changes differed between macrophages treated with Agaricus fractions and with LPS (unpublished data). These results suggest that the contribution of LPS contaminating Fractions B-4 and B-6 might be very small in macrophage activation.

In our recent paper (Sorimachi et al., 2001), Fractions A-4 and A-5 completely inhibited the occurrence of the cytopathic effect (CPE) induced by the western equine encephalitis (WEE) virus on VERO cells in vitro, and certain other fractions slightly inhibited CPE by WEE, herpes simplex or polio viruses. These results clearly show that Agaricus components, which affect the occurrence of CPE induced by viruses, differ from those which induce cytokine secretion by macrophages, and that Fractions A-4 and A-5 contain components which are not contained in Agaricus fruiting bodies.

Lignin derivatives (EP3) show the induction of TNF-α, IL-8 and NO secretion by macrophages (Sorimachi et al., 1999), and anti-viral activity for various viruses, including RNA and DNA viruses (Sorimachi et al., 1990b). Thus, EP3 has both functions. On the other hand, the different lignin derivatives (LS) show anti-viral activities (Suzuki et al., 1989b; Sorimachi et al., 1989), but do not show a significant effect on the cytokine secretion (Sorimachi et al., 1999). These characteristics of lignin derivatives differ from the characteristics of Agaricus components.

In addition, EP3 and LS show anti-tumor activity in vitro (Sorimachi et al., 1997). However, Agaricus fractions did not show anti-tumor activity in vitro in our preliminary experiments (unpublished data). Therefore, the fact that certain cancer patients apparently recover from cancer by taking Agaricus components as health food may be due to the activation of the immune system rather than the direct effect of Agaricus components on cancer cells in vivo. Thus, it is clearly shown that Agaricus blazei Murill contains certain components which activate macrophages contributing to the immune system in vitro.
References


(Received for publication, December 22, 2000 and in revised form, April 9, 2001)