Hypersensitivity Pneumonitis Induced by Spores of *Penicillium citrinum* in a Worker Cultivating Enoki Mushroom

Sumiko Yoshikawa¹, Kenji Tsushima¹, Tomonobu Koizumi¹, Keishi Kubo¹, Toshiko Kumagai² and Yoshitaka Yamazaki³

Abstract

A 47-year-old Japanese woman was admitted to our hospital with a 2-week history of dry cough and shortness of breath. She had been engaged in Enoki mushroom production for 22 years. Chest X-ray and chest computed tomography (CT) scan showed bilateral fine-nodular shadows and ground glass opacity. Bronchoalveolar lavage fluid demonstrated an increase of total cell counts with predominantly lymphocytosis. Pathological specimens obtained by video-assisted thoracoscopic surgery revealed alveolitis and noncaseating granuloma with giant cells. Lymphocyte stimulation test showed positive responses with Enoki mushroom, culture medium, and *Penicillium citrinum*. On double immunodiffusion test, a precipitation line was observed between patient’s serum and *Penicillium citrinum* antigen. She was found to have hypersensitivity pneumonitis caused by *Penicillium citrinum*. This is the first report of mushroom worker’s lung caused by *Penicillium citrinum*.

Key words: hypersensitivity pneumonitis, mushroom worker’s lung, Enoki mushroom, *Penicillium citrinum*

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Introduction

Hypersensitivity pneumonitis (HP) is an allergic immunoreactive disease caused by inhalation of a variety of environmental agents, and HP in mushroom workers is called mushroom worker’s lung (MWL). Recently, HP caused by inhalation of mushroom spores (1-4) has been reported in Japan. In other countries, thermophilic actinomycetes (5) and pyrethrum (6) have been reported as causes of MWL. *Flammulina velutipes* is called “Enoki” mushroom in Japanese and is a popular mushroom in Japan. Enoki has been cultivated in indoor environments to produce large quantities all year round. However, to our knowledge, there have been no previous case reports of MWL caused by inhalation of Enoki mushroom spores. On the other hand, *Penicillium* spp. have been shown to cause HP, such as humidifier/air conditioner lung (7), cheese worker’s lung (8, 9), and farmer’s lung (10). Here, we report a case of MWL induced by spores of *Penicillium citrinum*.

Case Report

A 47-year-old Japanese woman was admitted to our hospital with a 2-week history of dry cough and shortness of breath in August 2004. She had been engaged in Enoki mushroom production for 22 years, and her main job was picking and packing of Enoki mushrooms. She had no smoking history and no previous history of respiratory illness. On admission, her height was 163 cm and weight was 51 kg. Her temperature was 36.6 °C, blood pressure was 120/70 mmHg, pulse rate was 70 beats per minute with a regular rhythm, and respiration rate was 18 per minute. Bilarateral fine crackles were audible in the base of both lungs at the back. Results of laboratory examinations were within normal limits except for slightly positive C-reactive protein. However, the serum level of Krebs von der Lungen-6 (KL-6) was 607 U/ml (cut-off level, 450 U/ml), and serum level of surfactant protein-D (SP-D) was 196 ng/ml (cut-off level, 110 ng/ml) (Table 1). Arterial blood gas analysis indicated a PaO₂ of 61.3 torr in room air. The results of pulmonary...
function tests showed a reduction of vital capacity (VC) (43.9% of predicted). Chest X-ray revealed bilateral fine nodular shadows and ground glass opacity (Fig. 1), and chest computed tomography (CT) scan revealed reticulo-nodular shadow and ground glass opacity in both lung fields and linear atelectasis in the bilateral lower lobes (Fig. 2). Bronchoalveolar lavage (BAL) from the right middle lobe demonstrated an increase in number of total cells (71.4×10^4 /ml) with predominant lymphocytosis (41.6% of the total cells). The CD4/CD8 ratio of lymphocyte surface markers of the BAL fluid was 0.8. The specimens obtained by transbronchial lung biopsy (TBLB) showed lymphocyte infiltration in the alveoli. HP was suspected according to these results, especially based on chest X-ray and CT findings, but some questions have still remained. Linear atelectasis in the bilateral lower lobes on chest CT was incompatible with findings of typical HP, and alveolitis on TBLB was less specific to HP. These two findings might also be shown in non-specific interstitial pneumonia (NSIP). Furthermore, there has been no case report of acute HP in MWL according to Tsushima et al (11). Video-assisted thoracoscopic surgery (VATS) was necessary to correctly diagnose this case; it was performed after undergoing TBLB. The surgical specimens obtained by VATS revealed noncaseating granuloma with multinucleated giant cells. No fibrotic changes were found on elastica van Gieson staining (Fig. 3). These results suggested pathohistologically that she had HP.

To determine the causative antigen, an investigation of the workplace environment was performed using a standard type of swab on the air-conditioner, Enoki mushrooms, culture medium, and containers. Each sample was cultured in Sabouraud agar and potato-dextrose agar (PDA). In this investigation, _Penicillium citrinum_ was detected throughout the work environment (Fig. 4), while Enoki mushrooms and other fungi were not detected frequently. _Penicillium citrinum_ was also detected in the culture of the nasal cavity in this patient.

Lymphocyte stimulation test (LST) (12) was performed in peripheral blood (PB) and BAL fluid at a laboratory (Special Reference Laboratory Inc., Tokyo, Japan) without accompanying information about the patient’s clinical history. Enoki mushrooms, _Penicillium citrinum_ spores, and culture medium composed of six types of organic matter (rice sediment, wheat bran, bean husks, bean curd refuse, corn dregs and beet dregs) were selected for analysis. Enoki mushrooms, _Penicillium citrinum_ spores, and culture medium were kindly provided by the Japan Agricultural Cooperative Association in Nagano Prefecture. Lymphocytes in PB and

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**Table 1. Laboratory Examinations**

<table>
<thead>
<tr>
<th>Test</th>
<th>Value</th>
<th>Test</th>
<th>Value</th>
<th>Test</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>7480/μl</td>
<td>TP</td>
<td>6.8 g/dl</td>
<td>Na</td>
<td>139 mEq/l</td>
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<tr>
<td>RBC</td>
<td>5.1×10^12/μl</td>
<td>Hb</td>
<td>12.8 g/dl</td>
<td>AST</td>
<td>12 U/l</td>
</tr>
<tr>
<td>Hct</td>
<td>40.4 %</td>
<td>ALT</td>
<td>9 U/l</td>
<td>CRP</td>
<td>0.21 mg/dl</td>
</tr>
<tr>
<td>Plt</td>
<td>38.8×10^4/μl</td>
<td>ALP</td>
<td>191 U/l</td>
<td>SP-A</td>
<td>40.1 ng/ml</td>
</tr>
<tr>
<td>ESR</td>
<td>11 mm/hour</td>
<td>LDH</td>
<td>215 U/l</td>
<td></td>
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</tr>
</tbody>
</table>

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**Figure 1.** Chest X-ray showed bilateral fine-nodular shadows and ground glass opacity.

**Figure 2.** Chest CT scans showed reticulo-nodular shadow and ground glass opacity in both lung fields and linear atelectasis in the bilateral lower lobes.
Figure 3. A, B: Noncaseating granuloma with multinucleated giant cells (arrow) [A: H-E stain, ×10; B: H-E stain, ×40]. C, D: No fibrotic changes were found [C: elastica van Gieson, ×10; D: elastica van Gieson, ×40].

Figure 4. Investigation of the work environment. Colony counts of *Penicillium citrinum* in potato-dextrose agar (PDA). ① air-conditioner ②③④ Enoki mushroom ⑤⑥ culture medium ⑦⑧ container ⑨ dregs of culture medium

BAL fluid isolated from each sample by passage through a Ficoll-Conray gradient were suspended at a concentration of 1×10⁶ in 1 ml of PRMI 1640 medium containing 20% autologous plasma. Aliquots of 200 μl of suspensions were distributed into the wells of microtiter plates, and aliquots of 10 μl of diluted Enoki mushroom solution, *Penicillium citrinum* spore solution, and culture medium solution were added into each well. The solutions were prepared according to standard methods (3). Enoki mushroom solution and culture medium solution were diluted 50-, 250-, 1,250-, 6,250-, 31,250-, and 156,250-fold. *Penicillium citrinum* spore solution was diluted to 1,500 spores/μl, 300 spores/μl, 60 spores/μl, 12 spores/μl, 2.4 spores/μl, and 0.48 spores/μl. The stimulation index (SI) was calculated using a formula (3), and samples with SI values exceeding 400% were considered positive based on previous reports of MWL (11). Positive responses to *Penicillium citrinum* were observed in PB and BAL fluid (Table 2).

Double immunodiffusion test (Ouchterlony’s immunodiffusion test) was performed to detect precipitating antibodies. The antigens of Enoki mushrooms, *Penicillium citrinum* spores, and culture medium were prepared according to the standard methods (13). A large number of Enoki caps were placed in a case overnight and Enoki spores were collected from the bottom of the case the next morning. Equal volumes of phosphate-buffered saline (PBS) and glass beads 0.4 mm in diameter (Iuchi, Osaka, Japan) were added to the Enoki spores, and mixed for 30 seconds. This suspension was placed on ice for 1 minute in distilled water. This mixing procedure was repeated 10 times to break down the spores. Subsequently, the suspension was centrifuged at 14,000 rpm for 10 minutes, and was kept frozen at -70°C until use. *Penicillium citrinum* was grown on PDA medium at 27°C for 3 weeks, after which the mycelia were harvested and the spores were used for the experiments. Antigen preparation was performed in the same way as described for Enoki mushroom spores. Six components of the culture medium were homogenized and prepared for the experiment as described above. The gel was made of 60 ml of PBS, 0.4 g
of Bacto agar, and 0.2 g of Agar-EPI. The size of the glass filter was 10×10 cm, and the gel thickness was 1.5-2 mm. The diameter of each well was 3 mm, and the distance between each well was 7 mm. The concentration of each antigen solution was adjusted to 10 mg/ml. Aliquots of 8-10 μl of antigen solution or serum were added to each well, allowed to react for 24-48 hours, and the glass filter was washed and stained with Coomassie blue. To confirm that the assay system worked well as a control, the washed and stained antigen solution was adjusted to 10 mg/ml. Aliquotsof8-10μl of antigen solution or serum were added to each well, allowed to react for 24-48 hours, and the glass filter was rolled asanormalcontrol, and double immunodiffusion test (mean, 52.3 years), and had no allergic history, were enrolled as a normal control, and double immunodiffusion test was performed in their PB in the same way. No precipitating line appeared with Penicillium citrinum antigen, but no lines were present with the other antigens. These results suggested that Penicillium citrinum was the cause of HP in this case. Three healthy subjects, who were all women, nonsmokers, ranged in age from 50 to 55 years (mean, 52.3 years), and had no allergic history, were enrolled as a normal control, and double immunodiffusion test was performed in their PB in the same way. No precipitating line appeared with Enoki mushroom antigen, Penicillium citrinum antigen and culture medium antigen.

The patient was treated with 1 g of methylprednisolone intravenously for 3 days, then with 50 mg (1 mg/kg) of prednisolone daily. After treatment, her symptoms, findings of chest X-ray, and chest CT findings showed marked improvements. The dose of prednisolone was gradually tapered and discontinued in October 2004. She started working in the mushroom factory again after leaving our hospital. However, she suffered from dry cough and shortness of breath soon and stopped working. According to our advice, she now tries to keep her work environment clean and wears a mask during work. Subsequent to treatment, no respiratory symptoms have been observed.

**Discussion**

HP has some characteristic findings on laboratory examinations, such as elevated erythrocyte sedimentation rate, positive C-reactive protein, and leukocytosis in PB. However, these inflammatory changes were not observed in the present case. Takahashi et al and Tsushima et al suggested that KL-6 and SP-D were useful to diagnose HP (11, 14), and both were elevated in this case. Recently, HP caused by inhalation of mushroom spores, such as those of Cortinus shiitake (1), Pholiota nameko (2), Lyophyllum aggregatum (3), and Pleurotus eryngii (4), has been reported among workers in Japan. Chest X-ray and chest CT scans in mushroom workers with HP generally showed reticulo-nodular shadow and ground glass opacity, but in some cases, no abnormal shadows were observed on chest X-ray. Chest CT scans revealed linear atelectasis as well as the usual findings of HP in this case, and differential diagnosis from other types of interstitial pneumonitis was difficult.

Similar to these mushrooms, Flammulina velutipes (Enoki mushroom) is cultivated year round in an indoor environment, and the workers are always exposed to tremendous numbers of fungi, bacteria, and organic antigens, which can cause HP. Investigation of the workplace environment of the present case revealed that the workers were exposed to large amounts of spores of Penicillium citrinum, but not to spores of Enoki mushroom. The spores of both Enoki mushrooms and Penicillium citrinum measure about 2 to 3 μm. Thus, they are small enough to reach the bronchioles and the alveoli and can cause HP. To our knowledge, there have been no previous case reports of HP caused by inhalation of Enoki mushroom spores. On the other hand, there are about 30 species of Penicillium spp., some of which are known to cause HP. For example, Penicillium notatum is known to cause humidifier/air conditioner lung (7), Penicillium roqueforti and Penicillium verrucosum cause cheese worker’s lung (8, 9), Penicillium brevicompactum and Penicillium olivicolor cause farmer’s lung (10), Penicillium expansum causes HP in the home (15), Penicillium glabrum and Penicillium frequentans cause cork worker’s pneumonia (16), and Penicillium camembertii causes Salami worker’s lung (17). There have been no previous case reports of HP induced by inhalation of spores of Penicillium citrinum. HP caused by Penicillium spp. has no particular characteristics on chest CT scans different from the usual type of HP, and most cases show ground glass opacity.

Culture medium was also suspected as a possible cause of HP in this patient because it had six types of organic constituents-rice sediment, wheat bran, bean husks, bean curd refuse, corn dregs, and beet dregs. To determine if it is possible for them to cause HP, the size of each organic constituent was checked by microscopy. The results were as follows: rice sediment, 3-5 μm; wheat bran, 15-20 μm; bean husks, 120-150 μm; bean curd refuse, about 100 μm; corn dregs, about 200 μm; and beet dregs, about 150 μm. With the exception of rice sediment, it was not possible for the other five types of organic material to reach the bronchioles and alveoli.

LST has been used to determine the causative antigen in HP patients (4). A previous report suggested that stimulation index (SI) values exceeding 400% should be considered positive according to screening in HP patients (11), and we
used SI values exceeding 400%. However, there are some
problems whether this value is acceptable to all HP cases.
Positive responses were observed to Penicillium citrinum.
LST in asymptomatic colleagues without HP was not per-
formed this time. Therefore, it must be performed in the fu-
ture. The causative antigen was still unknown, and double
immunodiffusion test was performed; this test was very use-
ful to distinguish the causative antigen from non-causative
antigens. These results suggested that Penicillium citrinum
was the cause of HP in this patient. The inhalation of a
large amount of Penicillium citrinum spores probably led to
the occurrence of HP in our patient. Provocation challenge
was not performed. It was considered that the test might be
positive based on her respiratory symptom when she started
working again.
In conclusion, this is the first case of MWL induced by
spores of Penicillium citrinum. Penicillium citrinum should
be considered one of the causes of MWL.

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