Experimental Hypersensitivity Pneumonitis Induced by *Fusarium kyushuense* in Mice

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Abstract: Experimental Hypersensitivity Pneumonitis Induced by *Fusarium kyushuense* in Mice: Koichi Harada, et al. Department of Hygiene, Kumamoto University School of Medicine—A recently described *Fusarium* species, *Fusarium kyushuense*, was isolated from dead leaves of egg plant in a greenhouse where a female farmer who developed hypersensitivity pneumonitis (HP) with progressing lung lesions had been working. The freeze dried fungus was exposed to specific pathogen-free, 6-week-old female C57Black/6J mice under light ether anesthesia. Each mouse received 40 µl of the suspended fungal solution by dropping it onto its nostrils in 5 consecutive days a week for 4 wk. The control group received 40 µl of 0.1 M sterilized phosphate buffered saline. The mice were killed on the 4th day after the final exposure. The lung indices increased dose dependently in the fungus exposed mice groups. The specific IgG anti-*F. kyushuense* levels in sera of the high dose group were significantly higher than in the control group (P<0.05 by Ryan's multiple comparison test). Histologically, the lungs of both low and high dose groups showed signs of atelectasis with granulomatous lesions containing multinuclear giant cells and activated macrophages. This is the first report that mice developed HP induced by repeated exposure to freeze dried *F. kyushuense*. (J Occup Health 2000; 42: 124–129)

Key words: *Fusarium kyushuense*, Mice, Hypersensitivity pneumonitis, Egg plant, Greenhouse

Hypersensitivity pneumonitis (HP) is an inflammation of the lung induced by the repeated inhalation of various organic materials¹. A study of HP, experimentally induced by *Thermoactinomyces vulgaris* Tsiklinsky which is one of the most frequent causal antigens for farmer’s lung, showed signs of interstitial peribronchiolar and perivascular infiltration of mononuclear inflammatory cells with granuloma formation². *Fusarium* species (sp.) is well known as a causative fungal agent of bronchial asthma or wheezing³, ⁴, and is found on farms⁵. Some farmers who work in greenhouses where egg plants grow suffer from respiratory symptoms, such as sneezing, coughing, lacrimation and so on⁵. These symptoms are recognized as immediate type hypersensitivity⁶. In contrast to these patients with acute symptoms, a female farmer who grows egg plant in greenhouses in the southern part of Kumamoto City in Kumamoto prefecture, Japan, developed febrile and dyspnea symptoms of HP. HP also occurred in some male farmers who were stockbreeding calves, and *Fusarium* sp. was detected in the atmosphere of the stock yard when finely cutting wheat straw⁶. These reports indicated that *Fusarium* sp. is another candidate for the fungus which induces HP. In the process of disclosing causative organisms, we found a new *Fusarium* sp. called *Fusarium kyushuense* (*F. kyushuense*), in a greenhouse for egg plant growing⁷. This finding caused us to do a study on the HP induced by *F. kyushuense*. The purpose of the present study was to confirm whether HP was induced in mice by exposure to *F. kyushuense*.

Materials and Methods

Animal

Specific pathogen-free, 6-week-old female C57Black/6J mice, recognized as a strain of mice sensitive for the development of HP⁸, were purchased from an animal breeder (SLC Inc., Shizuoka, Japan). The mice were fed under specific-pathogen-free conditions, allowed food and water ad libitum, and were kept at a constant...
temperature of 22 ± 2°C and a relative humidity of 50–70%, and under a 12/12-h dark/light schedule. They received care in compliance with the Guide for the Care and Use of Laboratory Animals of the Research Center, Kumamoto University School of Medicine.

**Isolation and Identification of Fungus**

Dead leaves of the egg plant were collected from a greenhouse where a female farmer who developed HP was working. Some of the dead leaves were placed on a half strength of corn meal agar medium containing chloramphenicol in petri dishes. The petri dishes were incubated at room temperature. The fungi grown in the dish was transferred into the next petri dish. Single spore isolation was made under a microscope. After confirming the growth of the fungus on the medium, the fungus was transferred again into liquid medium (L.M.) in 500 ml Erlenmeyer flasks. The inoculated flasks were placed in a shaker rotating at 200 rpm at 25°C for a week. The pellets were collected by centrifugation at 3,500 g at 4°C for 5 min. The sediment was washed with sterilized physiological saline twice and then washed with sterilized distilled water three times. The washed fungal growth was collected by centrifugation and transferred into a sterilized petri dish and freeze dried. The fungus was identified as *F. kyushuense*, according to the description of Aoki and O’Donnell. The identification was confirmed by Japan Food Research Laboratories (6–11–10, Nagayama, Tamachi, Tokyo, 206-0025, Japan).

**Media**

Half strength corn meal agar: 8.5 g corn meal agar (Difco, Detroit, USA), 8.5 g agar (Wako, Osaka, Japan) in 1 liter distilled water. Corn meal agar: 17 g corn meal agar (Difco), 17 g agar (Wako) in 1 liter distilled water. L.M. (liquid medium): 10 g glucose (Wako), 5 g peptone (Difco), 3 g yeast extract (Difco), 3 g malt extract (Difco) in 1 liter distilled water. Sabouraud dextrose agar (Nissui, Tokyo, Japan): 65 g Sabouraud dextrose agar (40 g dextrose, 10 g peptone, 15 g agar) in 1 liter distilled water; pH 5.8. Phytone yeast extract agar: 40 g dextrose, 10 g phytone peptone (Becton Dickinson, Cockeysville, USA), 5 g east extract (Difco), 20 g agar (Wako), 50 mg chloramphenicol (Merck, Darmstadt, Germany) in 1 liter distilled water. Potato dextrose agar: 39 g potato dextrose agar (Difco) in 1 liter distilled water.

**Exposure of mice to the freeze dried fungus**

Two hundred mg of freeze dried *F. kyushuense* was suspended in 10 ml of sterilized 0.1 M phosphate buffered saline and sonicated at 20 kHz in a sonicator (Branson Sonifier cell disrupter 185, Danbury, Connecticut, USA) for a few seconds to homogenize the suspended solution. Each mouse received 40 μl of the suspended fungal solution by dropping it onto its nostrils with slight ether anesthesia. The high dose group received 800 μg of the freeze dried fungus suspended in 40 μl of 0.1 M phosphate buffered saline per mouse. Forty μl of the 5 fold diluted fungal solution was dropped on in the same manner as in the low dose treatment. The low dose group received 160 μg of the freeze dried fungus per mouse.

In the control group, mice were exposed to 40 μl of the sterilized phosphate buffered saline in the same manner. The negative control (normal animal) received 40 μl of saline to estimate the lung indices. The exposure treatment was performed on 5 consecutive days per week for 4 wk. The mice were killed 4 d after the final administration. The lungs were isolated from the mice and the body and lung weight were weighed to calculate the lung indices at the time of sacrifice. The lung indices were calculated as follows.

\[
\text{Lung Index} = \frac{\text{Organ wt}}{\text{body wt (test animal)}} - \frac{\text{Organ wt}}{\text{body wt (normal animal)}}
\]

The lung weight % were calculated as follows.

\[
\text{Lung Weight } \% = \frac{\text{Lung wt}}{\text{Body wt}} \times 100\%
\]

The blood was collected through the hepatic portal vein and left for a while in a refrigerator. The supernatant was taken as sera sample after centrifugation of the collected blood.

**Antibody (IgG) assay against *F. kyushuense***

IgG in the mice sera was determined by the usual enzyme linked immunosorbent assay (ELISA) method as follows. The freeze dried *F. kyushuense* antigen was dissolved in 0.1 M phosphate-buffered saline at a concentration of 1 mg/ml. 200 μl of the solution was left overnight at 4°C in each of the 96 wells of a microplate (Nunc-ImmuNo Plate MaxiSorp Surface, Nalge Nunc International, Denmark). Then the wells were rinsed with 0.1 M phosphate-buffered saline 3 times, and spread with 10% bovine serum albumin (Bovine Albumin Fraction V, Irvine Scientific Inc., Santa Ana, California, USA)-0.1 M phosphate-buffered saline overnight at 4°C. The wells were then rinsed with 0.1 M phosphate-buffered saline 3 times and 50 μl of the mouse sera diluted 1:10, 1:100, 1:1000, 1:10000 dilution in 1% bovine serum albumin-0.1 M phosphate-buffered saline overnight at 37°C. The wells were then rinsed with 0.1 M phosphate-buffered saline 3 times and 50 μl of peroxidase-conjugated anti-mouse IgG immunoglobulin (Goat Anti-Mouse IgG, F(ab’)_2, Fragment Affinity Purified, HRP Conjugated, ICN Biomedicals, Inc., Costa Mesa, California, USA) at 1:3000 dilution in 1% bovine serum albumin-0.1 M phosphate-buffered saline was put into each well for one hour at 37°C. The wells were rinsed with 0.1 M phosphate-buffered saline 3 times and 50 μl of the solution was left overnight at 4°C. Each well received 0.1 M phosphate-buffered saline 3 times and 50 μl of peroxidase-conjugated anti-mouse IgG immunoglobulin (Goat Anti-Mouse IgG, F(ab’)_2, Fragment Affinity Purified, HRP Conjugated, ICN Biomedicals, Inc., Costa Mesa, California, USA) at 1:3000 dilution in 1% bovine serum albumin-0.1 M phosphate-buffered saline was put into each well overnight at 4°C. Then 40 μg of o-phenylenediamine
dihydrochloride, 1.42 g of Na₂HPO₄ and 1.05 g of citric acid were dissolved in 100 ml of distilled water. 50 µl of the solution was blended with 5 µl of 30% H₂O₂ and put into each well, and the absorbance at 490 nm was measured in a microplate reader (Vmax Kinetic Microplate Readers, Molecular Devices Corp., Sunnyvale, California, USA).

**Histology**

A part of the isolated lung was fixed in 10% neutralized formalin and embedded in paraffin by the usual procedure. The packed tissue was treated by the usual method to prepare the slides. The 3 µm lung sections were stained with hematoxylin and eosine.

**Mycotic observation of the lung tissue from the infected mice (assay for lung colony forming units)**

Lung tissues were collected and homogenized in a sterilized phosphate buffered saline in an ice bath under sterile conditions. The lung homogenate was diluted serially and cultured into Sabouraud dextrose medium, Phytone Yeast extract agar medium, potato dextrose medium and half strength corn meal agar, and incubated at 25°C for a month.

**Statistical Analysis**

Among three groups (two exposed subgroups and one control group) significance was tested by one way analysis of variance (ANOVA); where the ANOVA showed significance at P<0.05, Ryan’s multiple comparison test (13, 14) was used to identify which subgroup or group was significantly different from the other subgroup or groups.

**Results**

F. kyushuense was isolated and identified from the dead leaves of egg plant collected from a greenhouse. Fig. 1 shows the morphology of conidia and macro conidia of F. kyushuense isolated from the dried egg plant leaves. F. kyushuense was characterized by the absence of chlamydospore, which was recognized to be the key morphological character distinguishing it from Fusarium sporotrichioides (7).

**Lung indices**

Table 1 shows that the average lung indices increased dose dependently in the mice groups exposed to freeze dried F. kyushuense. Both low and high dose groups also showed a statistically significant increase in the lung weight % when compared to the control group (Table 2).

**IgG in mice sera**

Figure 2 shows the level of specific IgG anti-F. kyushuense antibody in serum. The antibody level of the high dose group was significantly higher than that of the control group.

**Table 1.** Lung Index in the mice exposed to the freeze dried fungi

<table>
<thead>
<tr>
<th>group</th>
<th>Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1.041 ± 0.106</td>
</tr>
<tr>
<td>low dose</td>
<td>1.559 ± 0.085*</td>
</tr>
<tr>
<td>high dose</td>
<td>2.439 ± 0.383*</td>
</tr>
</tbody>
</table>

*Significantly different at P<0.05 from control by Ryan’s multiple comparison test. Each datum represents the mean (± 1 SD) for 5 mice.

**Table 2.** Lung weight (%) in the mice exposed to the freeze dried fungi

<table>
<thead>
<tr>
<th>group</th>
<th>Lung weight %</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.713 ± 0.049</td>
</tr>
<tr>
<td>low dose</td>
<td>1.033 ± 0.024*</td>
</tr>
<tr>
<td>high dose</td>
<td>1.601 ± 0.248*</td>
</tr>
</tbody>
</table>

*Significantly different at P<0.05 from control by Ryan’s multiple comparison test. Each datum represents the mean (± 1 SD) for 5 mice.
control group (P<0.05 from the control by Ryan’s multiple comparison test).

**Histology**

Histologically, the lungs of both low and high dose mouse groups given the *F. kyushuense* intranasally showed signs of atelectasis with granulomatous lesions (Fig. 3A, B). In granulomas multinuclear giant cells and activated macrophages were frequently observed (Fig. 3B, C). Infiltration of plasma cells containing Russell bodies was observed in the peribronchial space (Fig. 3D). There were no pathological changes in the lungs of the control group given the phosphate buffered saline intranasally (Fig. 3E, F).

**Mycotic observation of the infected lung tissue**

*F. kyushuense* did not grow in any culture media such as Sabouraud dextrose medium, phytone yeast extract, potato dextrose agar or corn-meal agar from the homogenized lung tissue infected by the fungus. In a positive control with the freshly suspended freeze dried *Fusarium kyushuense* infected by the fungus. In potato dextrose agar or corn-meal agar from the Sabouraud dextrose medium, phytone yeast extract, and family *Tuberculairiae*, and is thought to be one of the candidates causing HP in the farming areas examined in the present study. *F. kyushuense* exhibited an experimental HP in the laboratory mice. This is the first report detecting HP induced in mice as a result of repeated exposure to freeze dried fungus prepared from *F. kyushuense*.

In the present study, the internasally instilled *F. kyushuense* antigen induced HP. Hypersensitivity was characterized by a high lung index and high IgG-anti-*F. kyushuense* antibody levels in serum in the high dose group. Furthermore, the lung histology of low and high dose groups showed atelectasis with granulomatous lesions with multinuclear giant cells and activated macrophages. The IgG anti-*F. kyushuense* antibody levels had increased in the high dose group at four days after the final exposure to the antigen in the present study, but we did not perform the inhibition test to confirm the specificity of the IgG antibody level increased in the high dose group against *F. kyushuense*. Plotting the test is our assigned topic to be reviewed in detail in the future. Similar results have been reported for the increased prevalence of IgG-induced sensitization and HP, humidifier lung, in non-smokers exposed to aerosol under contaminated air conditions. An increase in the serum IgG level was also recorded during the course of sensitization with antigen suspension prepared from *Thermoactinomyces vulgaris*. Not only *Fusarium* sp. but also other fungi such as *Mucor*, *Eurotium*, *Acremonium*, *Alternaria*, *Cladosporium*, *Nigrospora*, *Penicillium* and *Trichothecium* sp. were identified in the dried leaves of egg plant in the present study. These fungi are also anticipated as pathogens of HP in workers who plant the vegetable.

Although serum IgE was not determined in the present study, development of lung lesions on the fourth day after the final treatment indicated that the mice developed HP in their lungs. This type of HP in the mice may partly be explained by humoral immune reactions since the specific IgG level was increased. The accumulation of plasma cells containing Russell bodies also indicates the involvement of a humoral immune reaction. In the present study, however, some farmers were found suffering from respiratory symptoms such as sneezing, coughing and lacrimation, recognized as an immediate type of hypersensitivity. This reminds us of the possibility of IgE-mediated allergy induced by not only *Fusarium* sp. but also other fungi species. Further study should be
done to clarify the IgE-mediated allergy in work places. Since the large influx of T lymphocytes in the bronchial alveolar lavage of patients with HP is another characteristic feature, the importance of cell-mediated hypersensitivity reactions should also be considered. Ito et al. have mentioned that there was a granuloma formation with a heavy accumulation of neutrophils in the alveolar space in the challenge phase with *Trichosporon cutaneum*\(^{19}\). Infiltration of neutrophils was also reported in the acute stage of human HP\(^{20}\). Accumulation of activated macrophages and granuloma formation were found in the lungs of the mice in the present study. Therefore the HP induced in the lung of the mice is considered to be influenced by cellular immune reactions\(^{18}\) also. Consequently the immunopathogenesis of HP has been considered to involve both humoral and cellular hypersensitivity to the invading antigens.

There was no recovery of viable *F. kyushuense* from the infected lungs of mice. This indicated that the host had controlled the fungal infection. We presume that alveolar macrophages in the infected lungs succeeded in degrading the injected fungal elements, and thereby eliminated fungal infection. Waldorf et al. have indicated that the prevention of mucormycosis required inhibition of fungal spore germination by alveolar macrophages\(^{21,22}\). The pulmonary defense against Aspergillosis depends on

![Lung histology of mice treated with *Fusarium kyushuense* (A, B, C, D) or with the phosphate buffer (E, F). Stained in Hematoxylin and Eosin. A: Atelectasis was observed in a mouse in the high dose group (×10). B: Granulomas containing several multinucleated giant cells (arrows) were observed. Infiltration of neutrophils was also noted (×66). C: Some macrophages in the granuloma contained needle-shaped crystals (×120). D: Many plasma cells containing Russell bodies were observed in the peribronchial space (×160). E, F: No pathological changes were observed in mice exposed to phosphate buffered saline (E: ×10, F: ×66).](image-url)
early killing of conidia by alveolar macrophages. Alveolar macrophages provide the first line of host defence against airborne infection with Aspergillus conidia, and the activation of these cells may be considered a preventive measure. After Aspergillus germinates and the hyphae invade the pulmonary tissue, polymorphonuclear neutrophilic leukocytes become the main effector cells involved, killing the mycelia of Aspergillus by secreting oxidative and non oxidative metabolites\textsuperscript{20}. We, therefore, propose that the host might degrade \textit{F. kyushuense} fungal elements directly by oxidative and by non oxidative metabolites derived from polymorphonuclear neutrophilic leukocytes. Further study might be needed to clarify this point.

In conclusion, we confirmed an effective method by dropping suspended freeze dried fungi onto the mice’s nostrils under light ether anesthesia to prepare the fungi sensitized animal model. We also confirmed HP in mice administered freeze dried \textit{F. kyushuense} from the histopathological study. The immunopathology of HP has been considered to involve both humoral and cellular hypersensitivity due to the increased number of activated macrophages, granuloma formation and the increased IgG antibody levels.

\textbf{Acknowledgment}: We thank Mr. Hiroshi Baba of the Food Institute in Japan for identifying the fungus as \textit{Fusarium kyushuense}, and Ms. Ayumi Nakamura and Ms. Yumi Nakamura for their technical support in the present study.

\textbf{References}


