

Original Article

## The Pathogenicity of *Stachybotrys chartarum*

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### Abstract

*Stachybotrys chartarum* is a dematiaceous fungus that is ubiquitous in our living environment. This fungus has long been regarded as non-pathogenic and its inhalation effect on humans has been scarcely studied. Recently, however, epidemiologic studies on acute idiopathic pulmonary hemorrhage in infants suggested that the fungus might be potentially pathogenic to humans. To determine the pathogenicity of this fungus, its interaction with the host defense system was studied using polymorphonuclear leukocytes (PMNs) and macrophages. Histopathological analysis of mice intratracheally injected with this fungus was also performed. The results disclosed that the conidia of *S. chartarum* were resistant to the antifungal activities of alveolar macrophages in terms of phagocytosis, killing and inhibition of germination. However, the conidia could not survive in the lungs of mice when injected intratracheally. Lavage fluid of mycelia that contained the dark slimy material coating the surface of conidia showed cytotoxic activity against macrophages and PMNs. Intratracheal injection of conidia in mice resulted in intraalveolar infiltration of PMNs. When using multiple injections during a 3-week period, strong eosinophilic infiltration into the proximal alveoli and perivascular tissues was observed. Our results suggest that inhalation of conidia may cause serious damage to the human lung, particularly when repeated.

**Key words:** *Stachybotrys chartarum*, pathogenicity, animal model, intratracheal instillation, eosinophilic infiltration, sick building syndrome

### Introduction

*Stachybotrys chartarum* is a dematiaceous fungus commonly found in soil, fodder, dwellings and the human living environment. This fungus is known to produce various toxins, such as satratoxins, stachybotrylactone, stachybotrylactam and atranons<sup>1-4</sup>, and sometimes causes poisoning of domestic animals such as horses when their feed is contaminated by toxic isolates. However, its pathogenicity to humans had been hardly examined until recently. Starting from 1993, outbreaks of acute idiopathic pulmonary hemorrhage in infants have been reported, and

exposure to this fungus growing in the living environment of the patients was suggested as the cause<sup>5-7</sup>. These episodes drew the attention of researchers to the relation between this fungus and human health. It is not yet clear whether the fungus caused the outbreaks, but the potential of this fungus to cause some human diseases is now being suspected.

To learn the pathogenicity of the fungus, its interaction with leukocytes and the effect of their culture filtrate and lavage of mycelia were studied. Mice were injected intratracheally with the fungus, and they were subjected to for histopathological examination.

### Materials and Methods

#### Isolates of *S. chartarum*

Twenty-one isolates of *S. chartarum*, collected

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from dwellings and environments such as soils and trees in Japan and China, were used. All isolates had been stored and maintained in the culture collection of the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University (IFM 41781-5, 41787-90, 52908-13, and 53635-40).

#### Growth temperature

The isolates were inoculated to potato dextrose agar (PDA: Difco Laboratories, Detroit, MI, USA) slants, cultured for two weeks at designated temperatures starting from 25°C, and the highest growth temperature was determined.

#### Cytotoxicity of lavage fluid and culture filtrates

Conidia of *S. chartarum* are generally coated with a dark slimy material that might have some biological activity when inhaled. This material was collected by lavage of mycelia, and the cytotoxicity of the lavage fluid as well as that of culture filtrates was examined against leukocytes. First, conidia of the fungus were collected. The 21 isolates were transferred to PDA slants (Ø30 mm × 200 mm), and cultured for 4 weeks at 25°C. Each isolate was washed in a total of 20 ml of RPMI 1640, and the supernatant was collected and centrifuged (1190 × g, 15 min) to collect the conidia. The final supernatant of each isolate was used as lavage fluid after sterilization with membrane filters (0.22 μm) (Millipore, Bedford, MA, USA).

The concentration of conidia (IFM 41788 and 41789) was adjusted to  $5 \times 10^5$ /ml in RPMI 1640 (Sigma Chemical, St. Louis, MO, USA). This suspension of conidia was dispensed into 24-well microplates (400 μl/well) (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), and they were incubated for 1, 3, 5, 7 or 14 days in a 5% CO<sub>2</sub> incubator at 37°C. Then each suspension was filtrated with a membrane filter for sterilization.

To examine the cytotoxicity of the culture filtrate and lavage fluid of each isolate, murine peritoneal macrophages and human polymorphonuclear leukocytes (PMNs) were used. Murine peritoneal macrophages were collected from BALB/c mice (8 weeks old, male, Charles River Japan Inc., Yokohama, Japan). The mice were anesthetized with ether (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and the brachial arteries were cut to collect blood. The mice were then sacrificed by cervical dislocation, and the peritoneal cavities were lavaged with a total of 10 ml of RPMI 1640. Peritoneal cells were collected, washed, and used as murine perito-

neal macrophages.

PMNs were collected from healthy volunteers. Heparinized peripheral blood was overlaid on Mono-Poly resolving medium (Dainippon Pharmaceutical Co., Ltd., Osaka) and centrifuged (400 × g, 20 min) to collect PMNs. These PMNs were washed with RPMI 1640 and used for the experiment. Serum was collected from the same donors and was stored at -80°C as autologous serum.

The concentrations of murine peritoneal macrophages and human PMNs were adjusted to  $4 \times 10^5$  cells/ml with RPMI 1640, and the suspensions were put into 96-well microplates (50 μl/well). Culture filtrates or lavage fluids were diluted with RPMI 1640 when necessary and 50 μl was added to each well to a final concentration of 5, 10, 30, or 50%. Then, fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA) and 10% autologous serum were added to macrophages and PMNs respectively, and the leukocytes were incubated for 24 hours in a CO<sub>2</sub> incubator at 37°C. Morphological changes of cells under each condition were observed with an inverted microscope (CKX41, Olympus, Tokyo).

#### Phagocytosis, killing and germination inhibition rate of conidia by alveolar macrophages

The antifungal activities of murine alveolar macrophages were determined. To collect murine alveolar macrophages, BALB/c mice (8 weeks old, male) were anesthetized with ether, blood was collected, and they were sacrificed by cervical dislocation. A catheter (24G, Insite-W, Becton Dickinson, UT, USA) was inserted into the trachea and fixed with silk thread. The lungs were washed through the catheter with a total of 10 ml of RPMI 1640. Bronchoalveolar lavage fluid was collected and centrifuged (320 × g, 10 min), and the pellets were used as alveolar macrophages. Their viability determined by Eosin Y dye exclusion test was more than 90%. Sera were separated from blood and used as autologous sera.

The conidia of isolates IFM 53635-7 and 53640 were collected as described above. These four isolates were selected based on the cytotoxicity of the lavage fluid — two isolates with high toxicity (IFM 53636, 53637) and the other two without toxicity (IFM 53635, 53640). The alveolar macrophage suspensions were put into 96-well microplates (100 μl/well) ( $1 \times 10^4$  cells/ml for phagocytosis;  $1 \times 10^5$  cells/ml for killing and germination inhibition rate). Then autologous serum was added at 10% and the

alveolar macrophages were incubated for 2 hours in a CO<sub>2</sub> incubator at 37°C. Non-adherent cells were removed by rinsing with RPMI 1640. Suspensions of conidia were put into the wells (100  $\mu$ l/well) ( $5 \times 10^4$  conidia/ml for the phagocytosis assay;  $1 \times 10^4$  conidia/ml for the killing and germination inhibition rate assay), 10% autologous sera were added, and cells were cultured at 37°C.

To assess phagocytosis, supernatants were removed from culture at 15, 30, 60, and 90 minutes, and macrophages were fixed and stained with Diff-Quik (International Reagents Corporation, Kobe, Japan). Phagocytosis was examined under an inverted microscope, and was determined by the following formula:

$$\% \text{phagocytosis} = (\text{phagocytizing alveolar macrophages} / \text{total number of alveolar macrophages}) \times 100$$

$$\text{Phagocytic index} = (\text{number of conidia phagocytized by alveolar macrophages} / \text{phagocytizing alveolar macrophages})$$

To examine the killing of conidia by alveolar macrophages, the conidia in each well were collected with distilled water after 3 or 6 hours of incubation, and the suspensions were spread on PDA plates and cultured at 25°C for 48 hours. Killing was evaluated by counting the colony forming units (cfu).

To examine the germination inhibition rate, the numbers of non-germinating conidia inside macrophages as well as the total number of ingested conidia after 8 or 24 hours of incubation with an inverted were counted under microscope. The germination inhibition rate was calculated from the following formula:

$$\text{Germination inhibition rate} = (\text{number of non-germinating conidia} / \text{total number of phagocytized conidia}) \times 100$$

A clinical isolate of *Aspergillus fumigatus* (IFM 49896) was used for comparison throughout this experiment.

#### Characterization of cytotoxic substance(s) in lavage fluid

For characterization of the cytotoxic substance(s) in lavage fluid, representative isolates (IFM 52909, 53637) were used because of the potent toxicity of their fluids.

Thermal stability of the lavage fluid was examined by heat-treatment using a heating block (DTU-1C, Tietech, Nagoya, Japan) at 37°C, 50°C (10, 30 and 60 minutes), and 100°C (10 and 30 minutes).

Solubility of cytotoxic substance(s) was determined as follows. The chloroform extract of 50

ml of lavage fluid was dehydrated with Na<sub>2</sub>SO<sub>4</sub> and dried using a rotary evaporator. The dried product was dissolved with dimethylsulfoxide (Wako Pure Chemical Industries, Ltd.) and the water-soluble fraction was collected.

Regarding molecular size, lavage fluid was separated according to molecular size by ultrafiltration method using Centriprep YM-3 (nominal molecular weight limit) (Millipore).

To determine the effect of each treatment, an appropriate amount of the treated fluid was added to a 96-well microplate containing murine peritoneal macrophages. The macrophages were cultured for 24 hours at 37°C, and their morphological changes were observed sequentially up to 24 hours.

#### Histopathological changes caused by lavage fluid and conidia

##### 1) Single-injection experiment

The histopathological effect by single injection of a mixture of conidia and lavage fluid was examined. The isolate showing the most cytotoxicity in lavage fluid was used in this study (IFM 53637). Conidia were cultured on PDA slants for three weeks at 25°C, and conidia and lavage fluid were collected as described earlier. The concentration of the suspension of conidia was adjusted to  $1 \times 10^4$  conidia/mouse, and the volume of the lavage fluid was adjusted accordingly.

Male 6-week-old ddY mice (n=9; Gokita Breeding Service Co., Ltd., Tokyo, Japan) were anesthetized by injection of ketamine and xylazine. Catheters (24G) were inserted into the trachea and the mixture of lavage fluid and conidia (25  $\mu$ l/mouse) was injected through them. A control group (n=9) received intratracheal injection of RPMI 1640.

Mice were sacrificed with ether on days 1, 3 or 7 post injection, and organs, i.e. lungs, liver, kidney and spleens, were removed. The organs were fixed with 10% (v/v) formaldehyde and histopathologically examined.

##### 2) Repeated-injection experiment

To find out the effect of longer exposure to *S. chartarum*, intratracheal injections were made repeatedly using 6-week-old male ddY mice. In brief, the concentration of suspension of conidia was adjusted to  $1 \times 10^5$  conidia/mouse, and the volume of lavage fluid was adjusted accordingly (n=8). The suspensions were injected intratracheally twice a week for three weeks. Control mice (n=7) received repeated intratracheal injections of RPMI 1640. For comparison, an

isolate of *Penicillium decumbens* (IFM 46582) was injected intratracheally based on the same protocol. The mice were sacrificed with ether on day 4 after the last injection, organs were removed, fixed with formaldehyde, and then histopathologically examined.

#### Histopathological effect by isolates with different cytotoxicities

To determine the histopathological effect by isolates with different cytotoxicities, two isolates, one highly toxic and the other non-toxic in lavage fluid, were selected. Conidia and lavage fluid were collected from each isolate, and their mixtures ( $1 \times 10^4$  conidia/mouse suspended in  $25 \mu\text{l}$  of lavage fluid) were injected intratracheally (6-week-old ddY mice,  $n=9$ ). IFM 53635 was used as non-cytotoxic isolate, and IFM 53637 as cytotoxic isolate. The mice were sacrificed with ether on days 1, 3 or 7 after the single injection, the effect on the lungs was examined histopathologically.

#### Effect of lavage fluid and/or conidia on the lungs

Conidia, lavage fluid and their mixture were given separately in order to determine their respective roles in the pathogenesis, and their effects were compared.

Seven ddY mice (6 weeks old, male) were intratracheally injected with conidia (IFM 53637:  $8 \times 10^4$  conidia/mouse) or lavage fluid ( $35 \mu\text{l}$ /mouse), or the mixture of conidia and lavage fluid. The mice were sacrificed with ether on days 1 or 3, and the lungs, livers, kidneys, and spleens were examined histopathologically.

In all experiments, the animals were kept in

an isolator, and food and water were provided *ad libitum*. All mice were cared for in accordance with the rules and regulations set out by the Prime Minister's Office of Japan. Animal protocols were approved by the Special Committee on Animal Welfare of Chiba University.

### Results

#### Growth temperature

Although the rate of growth varied among the isolates, the highest growth temperature was  $37^\circ\text{C}$  in all 21 isolates. No isolate was able to grow at  $38^\circ\text{C}$  or higher.

#### Cytotoxicity of lavage fluid and culture filtrates of the fungi (Fig. 1)

When the culture filtrate was added at 1-50%, murine peritoneal macrophages grew more extensively in a spindle shape than control. These morphological changes were more severe when the culture filtrate resulting from a long culture period was added. In contrast, the effect of lavage fluid varied depending on the isolates. In some isolates, lavage fluid promoted the growth of macrophages as seen in the culture filtrate. In other isolates, however, lavage fluid caused a similar growth of macrophages only at low concentrations (1-10%). At higher concentrations (30-50%), intracellular vacuoles or granules were formed in macrophages, and eventually the macrophages were destroyed, and none remained at 24 hours. Particularly strong cytotoxicities were seen in isolates IFM 52909, 53637, and 53639, and the peritoneal macrophages were destroyed within 24 hours when these lavage fluids were added at only 1%. As for PMNs, cells were swollen and destroyed within 2 hours after exposure to lavage fluid

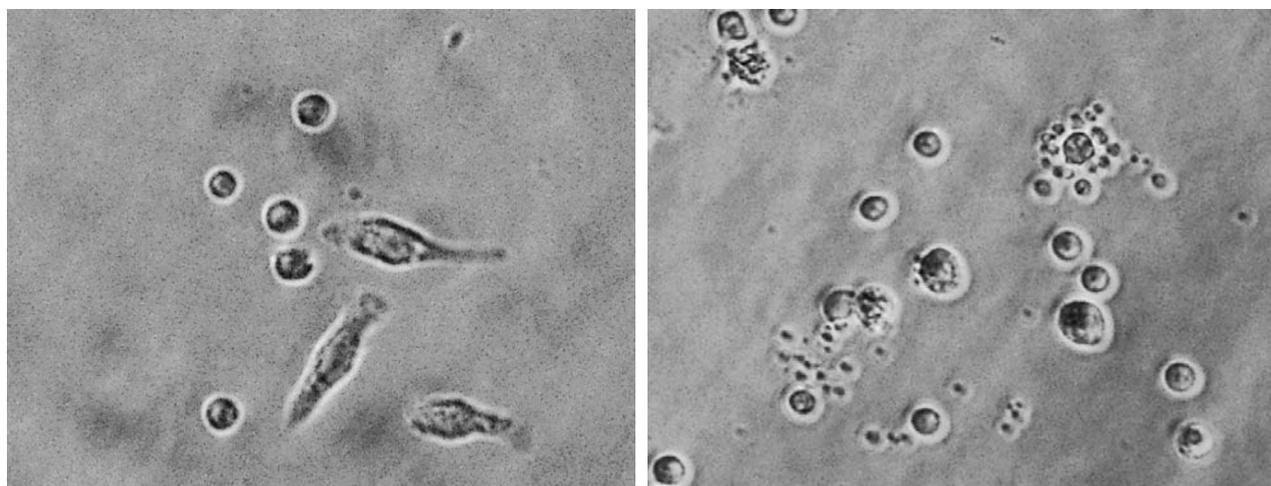


Fig. 1. Murine peritoneal macrophages exposed to the lavage fluid of *S. chartarum*. Mycelia of *S. chartarum* were washed with RPMI 1640 and the lavage fluid was collected. When lavage fluid of some isolates was added to murine peritoneal macrophages, they were destroyed within 24 hours (left, control; right, destroyed macrophages  $\times 200$ ).

regardless of the isolate.

**Phagocytosis, killing and germination inhibition rate of conidia by alveolar macrophages (Fig. 2)**

%phagocytosis of alveolar macrophages against *S. chartarum* conidia was 1.3-6.7% and 9.3-20.7% after 15 and 90 minutes, respectively. In contrast, it was much higher in *A. fumigatus*, reaching 42.7% at 15 minutes and remaining around 40% thereafter.

As for the phagocytic index, it was 1.0-1.3 after 15 minutes, and 1.1-1.5 in 90 minutes in *S. chartarum*. In *A. fumigatus*, it jumped up to 1.9 in 15 minutes.

In terms of killing, *S. chartarum* was slightly more resistant to the fungicidal activity of macrophages than *A. fumigatus*, although there was some variation among the isolates.

The germination inhibition rate of the conidia of *S. chartarum* varied significantly among the isolates (22.4-67.3%) at 8 hours. It reached 2.7-8.0% at 24 hours, just as low as that of *A. fumigatus*.

**Characterization of cytotoxic substance(s) in lavage fluid**

When lavage fluid was heat-treated for 30 minutes at 100°C, cytotoxicity decreased to 17%. When extracted by chloroform, lavage fluid showed cytotoxic activity in the chloroform soluble fraction. On the other hand, the water-soluble fraction did not cause any morphological change in macrophages, making them grow more remarkably than control at 10 and 30%. In terms of the fractionation of lavage fluid by molecular size, a fraction of smaller size ( $\leq 3$  kDa) showed

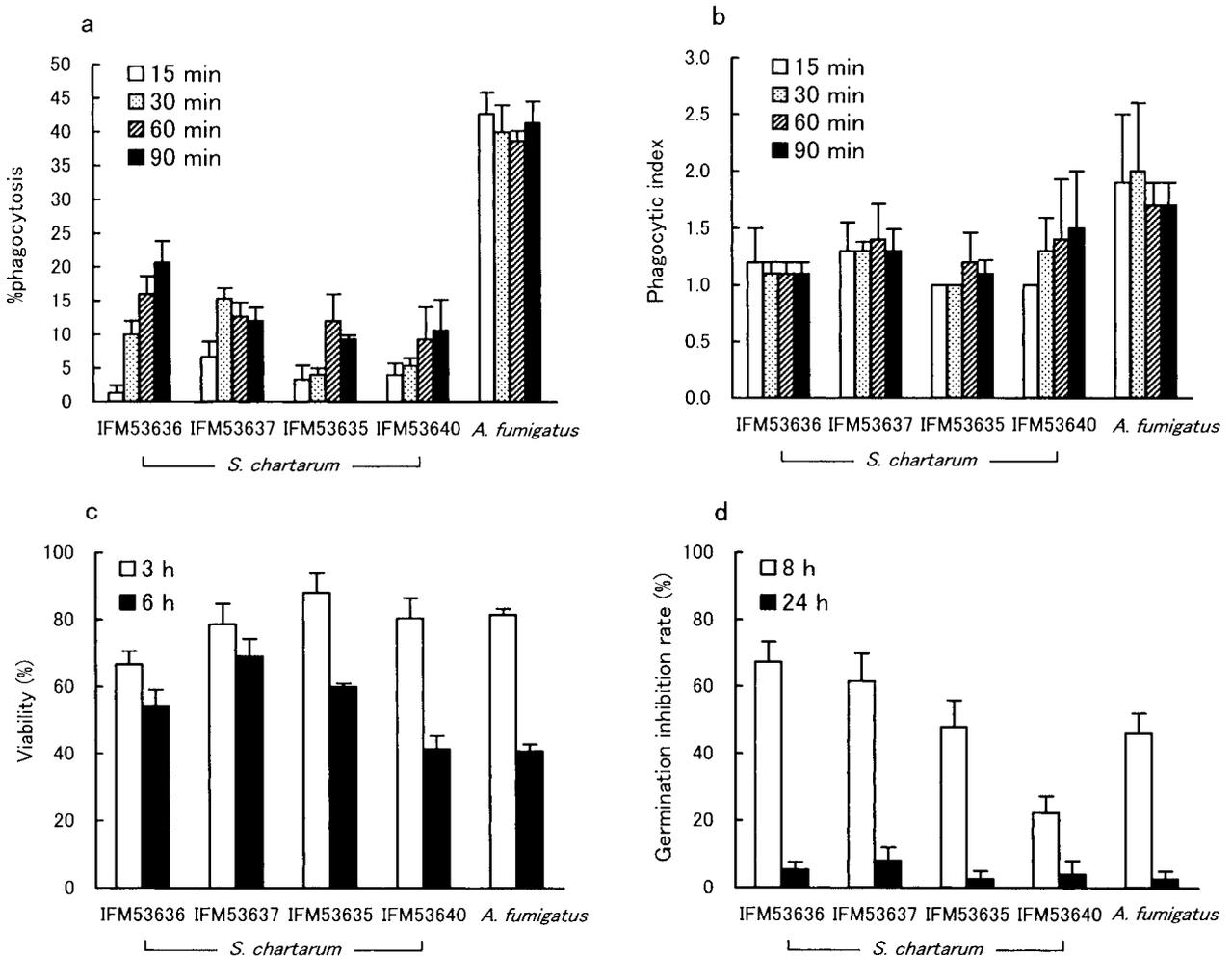


Fig. 2. Antifungal activity of alveolar macrophages against conidia of *S. chartarum*. Conidia of *S. chartarum* were co-cultured with murine alveolar macrophages, and the antifungal activity was determined. Phagocytosis was expressed by %phagocytosis (a) and phagocytic index (b), and was determined at 15, 30, 60 and 90 min. %phagocytosis of *S. chartarum* was significantly low compared with that of *A. fumigatus*. Conidia of *S. chartarum* were co-cultured with murine alveolar macrophages for 3 and 6 hours, and the number of viable conidia was determined by cfu. *S. chartarum* was resistant to the fungicidal activity by alveolar macrophages (c). The germination inhibition rate was determined after 8 hours, and significant variations among the isolates were seen. Most of the conidia completed germination after 24 hours (d).

strong cytotoxicity, destroying the macrophages at 5% or higher. In contrast, the cells remained uninjured when a fraction of larger molecular size ( $>3$  kDa) was added.

### Histopathological changes caused by lavage fluid and conidia

#### 1) Single-injection experiment (Fig. 3)

On day 1, inflammatory cells, mainly PMNs, infiltrated into the pulmonary proximal alveoli. By day 3, the inflammation had weakened, with the majority of inflammatory cells in the pulmonary alveoli changing to histiocytes. By day 7, inflammation had disappeared. Many conidia were found in the lung on day 1, but they then rapidly decreased, disappearing by day 7. None of the conidia in the lungs germinated throughout the course of the experiment.

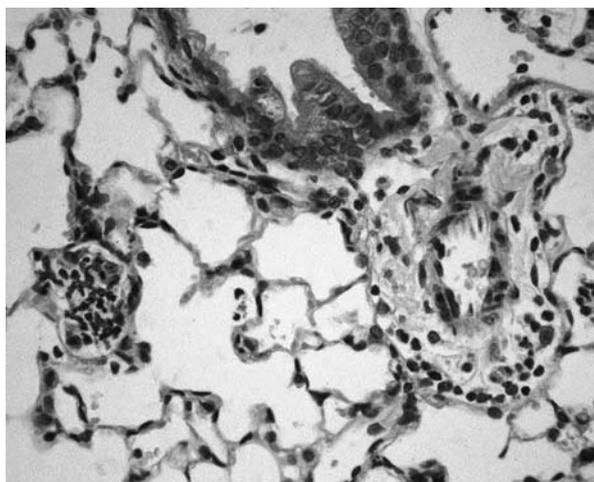
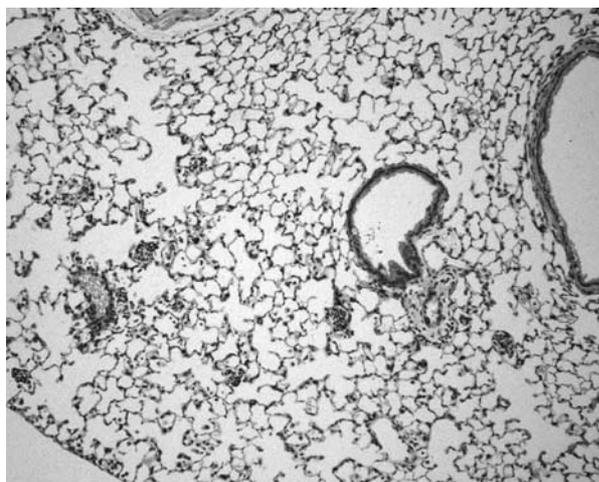


Fig. 3. Histopathological findings of the lung in the single-injection experiment. Mice were intratracheally injected with  $1 \times 10^4$  conidia/mouse and sacrificed on day 1. Inflammatory cells, mainly PMNs, infiltrated into pulmonary alveoli. (HE stain: left,  $\times 100$ ; right,  $\times 400$ )

#### 2) Repeated-injection experiment (Fig. 4)

Four days after the final injection of conidia, histopathological examination of the lung disclosed that eosinophils, accompanied by PMNs and histiocytes, infiltrated into the proximal alveoli and perivascular tissues. Multinucleated giant cells were also present. In contrast, no significant finding was seen in the mice that were exposed to *P. decumbens*.

The other organs, i.e. liver, kidney and spleen, were all normal in both experiments.

### Histopathological effect by isolates with different cytotoxicities

When mixtures of conidia and lavage fluid taken separately from the highly toxic and non-toxic isolates were injected, aggressive infiltrations of inflammatory cells, mainly PMNs, were seen in the proximal alveoli on day 1 in the

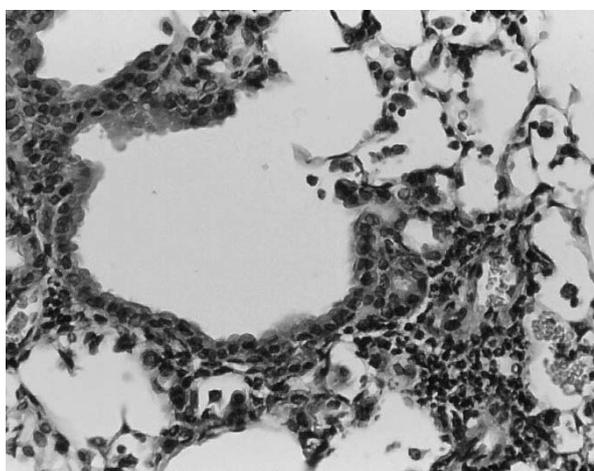
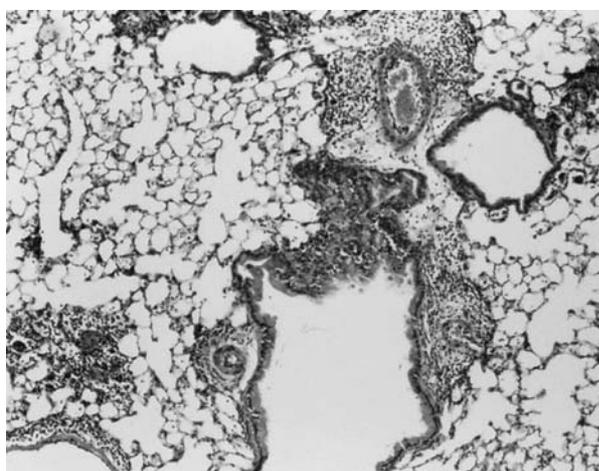


Fig. 4. Histopathological findings of the lung in the repeated-injection experiment. Mice were intratracheally injected with  $1 \times 10^5$  conidia/mouse twice a week for 3 weeks, and were sacrificed 4 days later. Recruitment of inflammatory cells, predominantly eosinophils, was evident around pulmonary arteries. (HE stain: left,  $\times 100$ ; right,  $\times 400$ )

highly toxic group. In contrast, the inflammatory response was much weaker in the non-toxic group.

#### Effect of lavage fluid and/or conidia on the lungs

To know the effect of conidia or lavage fluid per se, conidia, lavage fluid and their mixture were separately injected into mice, and the histopathological findings were compared. As seen in our experiments described earlier, acute inflammatory reaction was present on day 1 when the mixture of conidia and lavage fluid was given. When the conidia were solely injected, a similar response was seen. However, when the lavage fluid was given alone, no recruitment of inflammatory cells was observed, in stark contrast with the findings of the other groups.

#### Discussion

In this study we obtained the following results: 1) the highest growth temperature of the 21 isolates of *S. chartarum* was 37°C, which is high enough for growth in human bodies, 2) conidia of *S. chartarum* showed resistance to the host defense system by murine alveolar macrophages such as phagocytosis, germination block and killing, 3) the intratracheal inoculation of conidia caused strong inflammatory response by PMNs in a murine model, and they were eventually killed and removed from the lungs without germination, 4) repeated exposure of conidia to the lung caused eosinophilic infiltration into the proximal alveoli and perivascular tissues. Little has been known about the host defense system against *S. chartarum* and, to the best of our knowledge, this is the first study to demonstrate the interaction between *S. chartarum* and a host defense system of this kind.

Whether *S. chartarum* can cause infection in humans is still uncertain. To date, no case of human infection has been reported in the literature. From the aspect of the highest observed growth temperature, the fungus would seem to have the capacity to survive and grow in the human body. Furthermore, the fungus was rather resistant to the antifungal activity of leukocytes. However, in our animal studies, conidia failed to grow and were cleared from the lung. There was only one report in the literature that showed mycelial growth of *S. chartarum* in the lung of rats<sup>8)</sup>. However, as the animals of that study were just 4 days old, their immunological status would have been immature.

Although the precise defense mechanism against this fungus is not known, the result of our study suggests that the capability of *S. chartarum* to invade human lungs is low as long as the immunological status of the host is intact.

Although the fungus failed to invade lung tissues, it caused serious damage to the lungs by inducing another type of disease - a strong inflammatory response mainly by PMNs in alveoli and the peribronchiolar space. Repeated exposure resulted in an interesting histopathological finding in the lung, namely, eosinophilic infiltration into perivascular tissues and proximal alveoli. In contrast, only slight inflammation was seen when conidia of *P. decumbens*, one of the most common contaminants in the air of our living environment, was injected into the lung. This would indicate that *S. chartarum* carries the potential to cause serious acute inflammatory disease if inhaled.

To the best of our knowledge, it has as yet not been reported that *S. chartarum* caused significant eosinophilic infiltration in the lung. Recruitment of eosinophils is sometimes seen in certain mycoses such as coccidioidomycosis<sup>9)</sup>. However, in our case the infiltration of eosinophils was mostly limited to interstitial tissues around bronchioles and vessels. Although the mechanism of the eosinophilic infiltration in our model is not clear, the Th<sub>1</sub>/Th<sub>2</sub> balance might have been altered by the injection of *S. chartarum*. Considering the marked tissue inflammation, longer exposure could cause more serious damage in the lung, particularly around the arteries.

Although a few experiments have studied the effects of *S. chartarum* in the lungs of animals, the findings were inflammation by PMNs and histiocytes accompanied by granuloma formation<sup>10-12)</sup>, but the development of eosinophilic interstitial infiltration was not reported. Perhaps the differences in results depend on the species of mice or isolates of *S. chartarum* used. Another difference between their studies and ours was our unique method of inoculation. In the previous studies, intranasal instillation or intratracheal injection via tracheotomy was employed for endotracheal instillation<sup>10-16)</sup>, whereas we employed the intratracheal intubation method, which is not injurious to mice. Repeated injections cannot be performed once tracheotomy is done. With our safe method the mice were infected directly and repeatedly into the trachea. A few studies used repeated endotracheal instillation, but all of them employed the intranasal route<sup>10, 16)</sup>. Actually, by that method, the number of

conidia reaching the trachea is variable and unpredictable. In that sense, our method is much more reliable because the designated numbers of conidia are directly injected into the trachea, and this might have been the reason for the novel findings of our study.

The causative agent(s) of the strong inflammatory response in the lung is not known and the biological activity of the dark slimy material remains unanswered. However, the cytotoxic substance(s) in lavage fluid apparently plays a key role. Our study showed that the cytotoxic substance was thermotolerant, chloroform soluble, and a small molecular size ( $\leq 3$  kDa), but the active substance in the fluid has yet to be revealed. *S. chartarum* is known to produce various kinds of mycotoxins<sup>1-3)</sup>, and the cytotoxic activity of lavage fluid may be related to some of these toxins, such as satratoxins, stachybotrylactone and stachybotrylactam.

Although this fungus is known to be ubiquitous in our environment, analysis of fungal conidia in the air showed that it represents only a small percentage of all environmental fungi when conventional sampling method with the cfu counting system was employed<sup>17)</sup>. Because the detection of conidia of *S. chartarum* is known to be difficult by this method, the number of airborne conidia might have been underestimated<sup>18)</sup>. Recently developed genetic analyses have reported much higher sensitivity in the detection of *S. chartarum*<sup>19, 20)</sup>, and using this method a much larger number of conidia can be expected to be detected in our environment.

The significance of *S. chartarum* in our living environment has long been ignored. In fact, only as an animal feed contaminant has it received any attention. Our study shows the potential significance of this fungus as a biological hazard. In this sense, *S. chartarum* could be a cause of the sick building syndrome. Further studies of its pathogenicity are warranted. Such studies are now under way in our laboratory.

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