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ORIGINAL ARTICLE

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A murine model of pulmonary basidiomycosis by *Schizophyllum commune*

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Abstract *Schizophyllum commune* has recently emerged as a causative agent of human mycosis, but the details of its virulence are not yet known. To elucidate the pathogenicity of *S. commune*, a murine model of invasive pulmonary infection was established. ICR mice, not immunosuppressed or immunosuppressed by cortisone acetate, were infected with *S. commune* by intratracheal inoculation with agar beads containing the basidiospores. All immunosuppressed mice died within 2 weeks. Pathology examination revealed massive mycelial invasion into the lungs, penetration into adjacent vessels, and systemic dissemination, suggesting much higher virulence of this fungus than was previously estimated. This is the first murine model of pulmonary infection by *S. commune*, which we believe can be of assistance during subsequent investigations of this infection.

Key words *Schizophyllum commune* · Basidiomycosis · Animal model · Agar beads

Introduction

Schizophyllum commune is a ubiquitous basidiomycete that has long been regarded as nonpathogenic to humans. The first three reports of human infection by this fungus appeared in the literature during the 1950s, with *S. commune* being isolated from the nail,[1] cerebrospinal fluid,[2] and sputum.[1] However, the role of *S. commune* as a causative agent in these cases was not verified, so the significance of this fungus had remained unknown. The first confirmed case of infection, in which *S. commune* was isolated from the oral palate of a 4-month-old girl, was reported by Restrepo et al. in 1973.[3] Since then, *S. commune* has been reported to cause a number of human infections involving the lung[4–11] and paranasal sinus.[12–14] In 1999, twelve cases of this infection in Japan were reported and extensively analyzed.[15]

Evidently, the number of reported cases has been on the increase during the past 10 years, and some of the cases have involved invasive lesions. The most striking case was reported by Rihs et al. in which the fungus invaded the lung and brain of an apparently healthy man.[6] These clinical reports strongly suggest that the pathogenicity of this fungus may be much higher than is generally accepted, but details have remained unknown. Establishing an animal model of this infection would be an important step for the study of this disease.

An animal model is also desired with the view of developing effective treatment for the disease. Amphotericin B was tried in some cases, but its efficacy remained in doubt.[6] Susceptibility of this fungus to antifungal agents has not been demonstrated in vivo or in vitro. An optimal treatment method is therefore yet to be determined.

In this experiment we developed a murine model of invasive pulmonary infection with *S. commune* for the first time by intratracheal instillation of agar beads containing basidiospores. We believe that our model can contribute to a better understanding of this pathogen and to the development of therapeutic methods.

Materials and methods

Materials

Five-week-old female ICR mice (Charles-River, Japan Yokohama, Japan), housed in sterilized cages in a laminar flow hood, were used. Food and water containing tetracycline hydrochloride 300 mg/l (Wako Pure Chemical Industries, Osaka, Japan) were provided ad libitum. To facilitate the development of invasive basidiomycosis, 10 mice were

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immunosuppressed by subcutaneous injection of cortisone acetate (150 mg/kg) (Sigma Diagnostics, St. Louis, MO, USA) for seven consecutive days beginning 2 days prior to inoculation (day –2) to 4 days after the infection (day +4). Another 10 immunocompetent mice were given injections of sterile saline and served as controls.

A dikaryotic strain of *S. commune* (IFM 47009; Chiba University Research Center for Pathogenic Fungi and Microbial Toxicoses), originally isolated from a patient with allergic bronchopulmonary mycosis, was used. The basidiospores, which had been stored at 4°C in sterile water, were cultured on potato dextrose agar (PDA) plates (Difco Laboratories, Detroit, MI, USA) for 14 days at 30°C under a 12-h light-dark cycle to support the growth of basidiocarps; the basidiospores were collected immediately before each experiment. The viability of basidiospores was 98% when determined by counting the colony-forming units (CFUs) on PDA plates.

Methods

Schizophyllum commune basidiospores embedded in agar beads were inoculated intratracheally into mice to cause invasive pulmonary infection. Agar beads containing basidiospores were made based on the methods of Nawada et al.[16] and Cash et al.[17] In brief, collected basidiospores were washed and resuspended in *d*-H_2O at a concentration of 2×10^8 spores/ml. Microscopic examination disclosed spindle-shaped, single-cell basidiospores with no clumping. The suspension was mixed with the same volume of 7.8% PDA at approximately 50°C and was dripped immediately into heavy mineral oil (Sigma Diagnostics), which had been kept at 50°C in a flask. Immediately after vigorous stirring with a magnetic bar, the flask was cooled with crushed ice. This procedure solidified the agar droplets into beads of 150–200 mm diameter. The oil-bead slurry was washed twice with 0.5% sodium deoxycholate in saline and then washed with sterile saline. The agar beads were resuspended in sterile saline at a concentration of 2×10^6 cfu/ml and were used for inoculation. Agar beads without basidiospores were also prepared with PDA solution, as described above. To confirm the viability of the spores in the agar beads, the beads were incubated at 35°C for 24 h. When examined under a microscope, most of the spores were shown to have grown into the hyphal form.

Each mouse was anesthetized with an intraperitoneal injection of pentobarbital 60 mg/kg (Abbott Laboratories, North Chicago, IL, USA). Inhalation of diethyl ether was also used when needed. The trachea was exposed aseptically by a ventral cervical skin incision. A 50-µl aliquot of the agar bead suspension, containing 1×10^5 spores in 1×10^4 agar beads, was injected into the trachea with a 27-gauge needle, and the wound was closed with surgical glue. Agar beads without basidiospores were also used in some animals for comparison.

Mice were checked for activity and survival at least once a day. The cumulative 3-week mortality was determined for the survival study. After 21 days all mice were sacrificed and examined histopathologically. The mice that died before day 21 were also examined.

To determine the amount of CFUs of *S. commune* in the lungs, three mice were sacrificed on days +1, +3, +5, and +7 (in the nonimmunosuppressed group, additional mice were sacrificed on days +14 and +21). Each right lung was homogenized with a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland) for 30–60 s, after which the homogenate was seeded on PDA plates at 10-fold dilutions. The plates were incubated at 30°C, and the CFUs were counted 48 and 72 h later. The number of viable cells was expressed in log (cfu/lung).

The left lungs were fixed in 10% formaldehyde, and the sections were stained with periodic acid-schiff (PAS), Grocott, or hematoxylin-eosin (H&E) stain for the pathology study.

Statistical analysis was performed with Student's *t*-test or the log-rank test using Prism 2.0b (Graphpad Software, San Diego, CA, USA).

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 Committee on Animal Welfare of our institution.

Results

Survival

When the immunosuppressed mice were given the agar beads with spores, the animals started to die on day +7, and all the mice died by day +12. In contrast, none of the nonimmunosuppressed mice had died by day +21. To determine the direct effect of the inoculation of agar beads, beads without spores were injected intratracheally into immunosuppressed mice. No mice died from the procedure, indicating that the intratracheal inoculation of agar beads per se has no serious effect on the health of mice (Fig. 1).

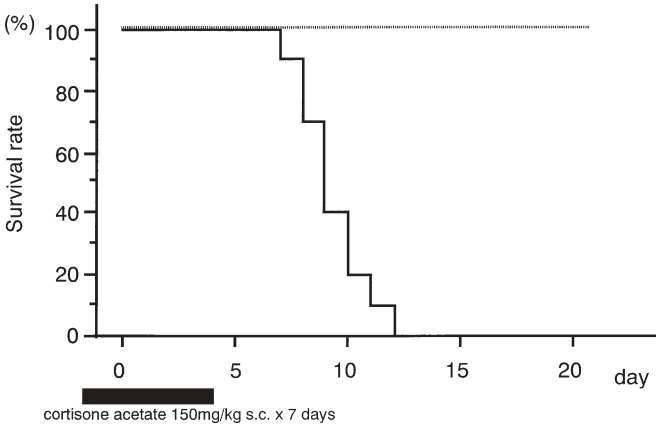

Fig. 1. Cumulative mortality of *Schizophyllum commune*-infected mice. All of the animals immunosuppressed with cortisone acetate (*solid line*) died by day 13, whereas none of the immunocompetent mice died (*dotted line*). For immunosuppression, mice received cortisone acetate at 150 mg/kg s.c. for 7 days