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Use of a Mouse Model to Evaluate Clinical and Environmental Isolates of *Sporothrix* spp. from the Largest U.S. Epidemic of Sporotrichosis

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Five clinical and 69 environmental isolates from the largest U.S. epidemic of sporotrichosis were evaluated in NYLAR male mice following intravenous injection of 5×10^6 to 2×10^8 conidia per mouse. The clinical isolates and eight environmental isolates produced 100% mortality in groups of three mice each between 12 and 24 days after injection. These virulent isolates grew at 37°C, were dematiaceous by virtue of melanin (melanized) on permissive media (e.g., potato dextrose agar), produced ovoid conidia borne sympodially on lateral conidiophores and pleurogenously about the main hyphal axis, and were identified as *Sporothrix schenckii*. Two melanized environmental isolates that grew at 35°C but not at 37°C were not virulent and had subtle morphological differences from *S. schenckii*. The remaining environmental isolates were not melanized, were not virulent, and were not *S. schenckii*; five were identified as *Ophiostoma stenoceras* and the remainder were identified as *Sporothrix* spp. Quantitative organ cultures revealed that clinical isolates grew exponentially in livers and testes, in contrast to an isolate of *O. stenoceras* that was eliminated from liver, lung, and spleen but that persisted in the testes throughout the 14-day sample period. This model helped to confirm the identification of *S. schenckii* isolates obtained from the environment.

The largest outbreak of sporotrichosis reported in the United States occurred in the spring and summer of 1988 and involved 84 people residing in 15 states, with more cases (25 cases) occurring in New York than in any other state (2, 7). All cases were associated with sphagnum moss derived from a single source in Wisconsin. Twenty-one clinical isolates of *Sporothrix schenckii* and 69 environmental isolates of *Sporothrix* spp. were recovered during a study of the epidemic (7). The clinical and environmental isolates were compared with respect to their macroscopic and microscopic morphologies, ability to grow at 37°C, ability to convert to a yeast phase, and exoantigen reaction with antisera to *S. schenckii*. Each environmental isolate was placed into one of six arbitrary groups on the basis of its macroscopic and microscopic morphologies. The environmental isolates suspected of being *S. schenckii* were those in group I. These isolates produced dematiaceous colonies and sleeves of melanized conidia on potato dextrose agar (PDA). None of the other environmental isolates (groups II to VI) produced dematiaceous colonies, nor did they produce sleeves of melanized conidia on PDA. Group II environmental isolates produced perithecia and ascospores on PDA and were identified as *Ophiostoma stenoceras*. Group III to VI isolates were characterized on the basis of minor differences in their colony morphologies (7).

We summarize here the results of virulence studies with all 69 of the environmental isolates of *Sporothrix* spp. and *O. stenoceras* and with 5 representative clinical isolates of *S. schenckii* from the epidemic using a mouse model of intravenous infection with conidia. The clinical isolates of *S. schenckii* and the environmental isolates in group I that grew at 37°C were equally virulent in mice, with 100% mortality obtained between 12 and 24 days after injection. No other environmental isolates, including *O. stenoceras*, appeared to be virulent. Thus, the animal model provided useful data to support the identification of *S. schenckii* isolates from nature associated with the epidemic of sporotrichosis.

MATERIALS AND METHODS

Fungi. Clinical isolates from the epidemic were received as reference cultures by the Laboratories for Mycology of the New York State Department of Health between June and October and were accessioned in the culture collection. They were maintained on PDA (Difco, Detroit, Mich.) slants at 4°C. Five representative isolates (isolates M537-88, M647-88, M665-88, M673-88, and M923-88) were selected for animal experiments. These isolates have been characterized previously (7). Environmental isolates were obtained from sphagnum moss or material contaminated with moss (62 isolates) by a direct plating technique; but isolates were also obtained from dirt (1 isolate), water (1 isolate), and evergreen seedlings (5 isolates) as described previously (7). These isolates were maintained in a sterile water culture collection (7). *O. stenoceras* M674-88 was originally sent as a suspected culture of *S. schenckii* isolated from the toenail of a patient with no signs or symptoms of sporotrichosis. The isolate did not appear to be causing disease.

Inoculum preparation. Cultures were grown on PDA slants for seven days at 30°C. Colonies were flooded with sterile, 0.85% saline (SPS) while gently rubbing the surface growth with a pipet tip. The resulting suspensions were collected with a pipet and filtered through a sterile paper wipe (Kimwipes; Kimberly-Clark, Roswell, Ga.) contained in a funnel. This resulted in suspensions of conidia devoid of hyphal fragments. The conidia were sedimented at 1,400 × *g* for 10 min and were resuspended in SPS to the desired hemacy-

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tometer counts, ranging from 2.5×10^6 to 1×10^9 conidia per ml.

Animal inoculations. Male NYLAR mice were used; they weighed 18 to 20 g when the experiments were initiated by the inoculation of conidia in 0.2-ml volumes into a lateral tail vein. Animal procedures were approved by the Institutional Animal Care and Use Committee. For mortality studies, mice received 6×10^5 to 2×10^8 conidia each. Strains that were subsequently determined to be avirulent produced sufficient conidia to yield an inoculum of $\geq 10^7$ conidia per mouse. Thus, avirulence was not associated with the lower range of the inoculum concentration. Groups of three mice each were inoculated with each fungal isolate, including the 5 representative clinical isolates and all 69 environmental isolates. Mice were examined once each day for the first 30 days and at regular intervals for up to 6 months. Moribund mice were euthanized. For histological study, selected organs were fixed in 10% formalin and adjacent sections were stained with hematoxylin-eosin or Gomori-Grocott methenamine silver. For quantitative organ cultures, groups of three mice each received 10^7 conidia of either *S. schenckii* (clinical isolate M537-88) or *O. stenoceras* (M674-88). Immediately after inoculation, at 2 days, and at 1 and 2 weeks after inoculation, three mice from each group were sacrificed; and the lungs, livers, spleens, and testes were removed for quantitative organ culture for fungi. Organs were placed in preweighed plastic bags (Whirl-Pak; Nasco, Fort Atkinson, Wis.), weighed, and then homogenized in 1.0-ml volumes of SPS as described previously (18). Serial tenfold dilutions of organ homogenates were made in SPS, and 0.2 ml was spread onto Mycosel agar (BBL, Cockeysville, Md.) plates. The dilutions that were plated were adjusted to account for the expected concentrations in each case. Qualitative organ cultures for the presence of fungi were done in selected animal experiments by using the homogenization technique described above, but without subsequent dilutions.

RESULTS

All five of the clinical isolates of *S. schenckii* were virulent in the mouse model, with the mean day of death in each strain ranging from 12.7 to 22.3 days after inoculation. Equally virulent were all eight group I environmental isolates that grew at 37°C, with mean days of death ranging from 13.0 to 20.7 days after inoculation. The infections established by both the clinical and the eight environmental group I isolates were uniformly fatal, with death or sacrifice of moribund animals occurring in all groups for all animals. The two environmental group I isolates that grew at 35°C but not at 37°C were not virulent in mice. None of the other isolates from any of the other environmental groups (groups II to VI) produced fatal infections in mice (Fig. 1).

Qualitative cultures of lungs, livers, spleens, testes, and brains from representative moribund mice infected with either clinical or virulent environmental group I isolates were grossly positive for *S. schenckii*. Microscopic examination of Gram-stained smears of homogenates of the organs or of Gomori-Grocott methenamine silver-stained histologic sections of portions of such organs revealed numerous oval and elongate (cigar-shaped) budding yeasts characteristic of *S. schenckii*. The in vivo morphologies of the two groups of isolates were indistinguishable.

Subcutaneous, nodular lesions were common on the legs, feet, and tails of mice infected with either clinical or virulent environmental group I isolates. Occasionally, such lesions were seen on the tails of mice injected with isolates of *O. stenoceras*. Colonies were recovered from the tail of one mouse inoculated with *O. stenoceras* M674-88 when the mouse was sacrificed 85 days after inoculation. Inguinal lymph nodes from two other mice inoculated with an environmental isolate of *O. stenoceras* were culture positive for the fungus when the animals were sacrificed 52 and 98 days after inoculation; the livers and testes of these animals were culture negative. Additionally, one environmental group V isolate was recovered from the inguinal lymph nodes of one mouse when they were cultured 52 days after inoculation. No other organs were culture positive.

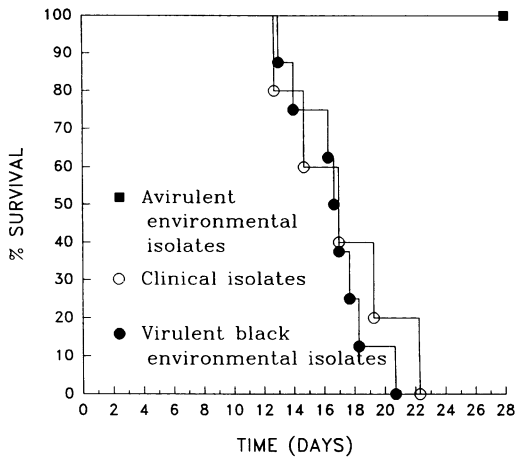

FIG. 1. Survival of mice following intravenous inoculation of conidia of *Sporothrix* species from clinical and environmental sources associated with the 1988 epidemic of sporotrichosis. Solid square, nonpigmented (groups II to VI) environmental isolates of *Sporothrix* spp., including the anamorph of *O. stenoceras* and two pigmented (group I) environmental isolates that did not grow at 37°C. Each animal received $\geq 10^7$ cells; open circles, clinical isolates of *S. schenckii*; closed circles, pigmented (group I) environmental isolates of *S. schenckii* capable of growth at 37°C. For the last two groups, mice received $\geq 5 \times 10^6$ cells each. Datum points represent the means for three mice.

The results of the quantitative organ culture experiments are shown in Fig. 2. Exponential growth of *S. schenckii* M537-88 was supported in the livers and testes of intravenously inoculated mice. The greatest fungal density of growth was achieved in the liver; the greatest relative increase in cell number was achieved in testes; the growth rate during the exponential increase was approximately the same in these two organs. Fungal counts of M537-88 decreased by approximately 2 log units in the lungs and spleens, but they persisted during the 2-week sample period. In contrast, an exponential decrease in *O. stenoceras* M674-88 cell numbers occurred in the lungs, livers, and spleens of experimentally infected mice. The rate of fungal killing was rapid, extending over 8 log units in the 2-week period. Fungi persisted in the testes of these animals over this period.

DISCUSSION

Our previous mycological study of 21 clinical and 69 environmental isolates associated with the largest U.S. epidemic of sporotrichosis revealed a group of eight environmental isolates that grew at 37°C and that were morphologically indistinguishable from *S. schenckii* (7). Results of

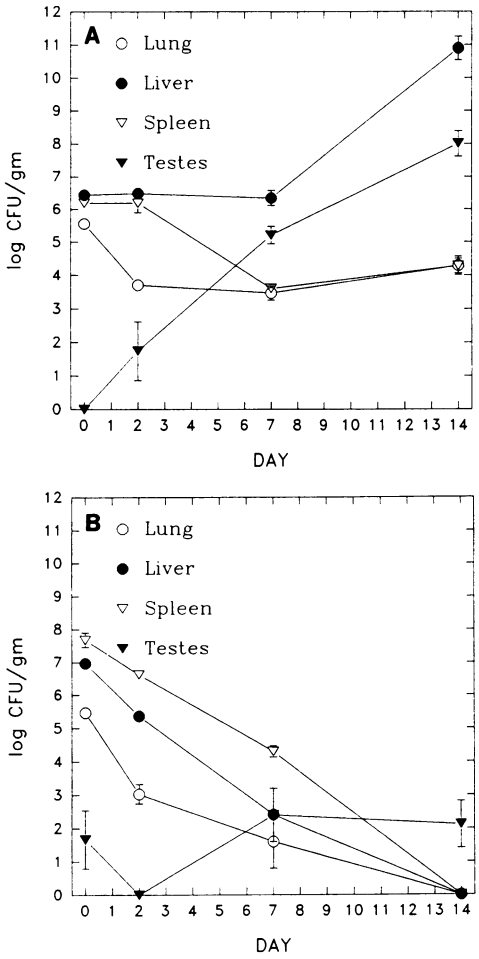

FIG. 2. Quantitative cultures of organs of mice infected with either *S. schenckii* M537-88 (A) or *O. stenoceras* M674-88 (B). Each mouse was inoculated intravenously with 10^7 cells. On the specified days, groups of three mice per fungus were sacrificed and homogenates of organs were plated individually to determine the number of CFU per gram of tissue. Datum points represent the means ± standard errors for three samples.

the present study demonstrate that these eight isolates, all from sphagnum moss associated with the epidemic, are virulent and are capable of producing uniformly fatal infections in mice. Hence, they could be representatives of the same population that produced the 1988 epidemic.

In our previous study, the clinical isolates of *S. schenckii* produced colonies and oval conidia on PDA that were dematiaceous by virtue of melanin (melanized), that all grew at 37°C, and that all converted to a yeast phase in vitro. These characteristics were shared by 8 of 10 environmental isolates (i.e., group I); the two remaining environmental group I isolates differed by their inability to grow at 37°C, although they grew at 35°C. All of the environmental group I isolates were identified as *S. schenckii* in the previous study (7).

Interestingly, the two isolates that did not grow at 37°C did not cause death in mice. Reevaluation of these two isolates revealed the following. One produced all of the microscopic characteristics associated with *S. schenckii*, including oval sympodial conidia in rosettes and the development of sleeves of melanized conidia. However, the isolate produced abundant clavate sympodial conidia atypical of *S. schenckii*. The other isolate represented an even more difficult situation because it was morphologically consistent with identification as *S. schenckii* in every respect except the absence of sleeves of melanized conidia. Both isolates converted to a yeast phase at 35°C. Both produced melanized colonies on PDA when they were initially isolated, but both lost degrees of melanization upon repeated transfer. Additionally, the DNA types of these two isolates have been found to be different from those of the virulent isolates (3). Without the use of animal virulence or DNA typing, the differentiation of these two isolates from *S. schenckii* is exceedingly difficult.

Since many of the morphologically similar nonpigmented environmental isolates of species other than *S. schenckii* (groups II to VI; see reference 7) also grew at 37°C and converted to a yeast phase in vitro, we thought that it was important to test the virulence potential of these isolates in mice. None of these isolates produced fatal infections in mice, even at concentrations of $\geq 10^7$ cells per mouse.

Animal passage of the isolates did not appear to affect the pigmentations of the nonpigmented colonies. Environmental group II to VI isolates continued to produce nonmelanized colonies in culture after recovery from inoculated mice. This included the strains of *O. stenoceras* that were recovered after 98 days. The eight virulent group I isolates were recovered as melanized colonies from mice. One isolate produced dematiaceous colonies with white sectors with reduced numbers of conidia when it was grown from infected kidneys.

This appears to be another example of the association between melanin and virulence in the dematiaceous fungi. The previous example from this group is *Wangiella dermatitidis* and the association of pentaketide melanin with virulence in experimental infections (5, 6, 8). The melanin in *S. schenckii* has not been characterized.

Mackinnon et al. (12) used direct plating and animal inoculation techniques to isolate *S. schenckii* from plant debris and soil in Uruguay. They also noted a correlation between dematiaceous pigmentation (on cornmeal agar) and virulence in mice. Isolates that produced characteristic oval or triangular conidia and that were pigmented on cornmeal agar produced orchitis following intratesticular injection in guinea pigs or periorchitis and lesions along the tail vertebrae and feet following intraperitoneal inoculation in mice. They noted that isolates of *Graphium* and *Ceratocystis* and strains resembling *S. schenckii* were more commonly isolated, but that these fungi did not produce pigmented colonies on cornmeal and did not produce characteristic lesions in animals, although some could be recovered from spleens up to 1 month after inoculation. They summarized that "*S. schenckii* possesses a definite virulence which seems to be characteristic of the species" (12). We concur with this. Findlay and Vismer (9) compared five sporothrixlike isolates from environmental sources associated with patient infections with five isolates from the patients. They noted a difference in pathogenicity between the environmental and the clinical isolates following intratesticular injection in rats. They commented on the relative avirulence of the environmental strains. This decreased virulence correlated with a failure of the environmental isolates to develop pigmented conidia in vitro (9).

Mariat (13) has described fatal infections with *Ceratocystis stenoceras* in hamsters and in mice with histopathology similar to that in mice with experimental infections with *S. schenckii*. He showed that growth of the inoculum at 35°C and animal passage enhanced the virulence of the species. Cultures produced perithecia prior to but not after animal passage. He referred to the strains that had lost the ability to

produce perithecia as mutants (13). Taylor (16) examined *S. schenckii* and the *Ceratocystis* species *C. minor*, *C. montia*, *C. multiannulata*, *C. narcissi*, *C. nigrocarpa*, *C. perparvispora*, and *C. pilifera* in an animal model of infection. Mice were inoculated intraperitoneally with hyphal fragments in sterile saline containing gastric mucin. Mortality rates of 20 to 100% were obtained in groups of animals inoculated with *S. schenckii* and *C. montia*, but not with the other species of *Ceratocystis* (16).

Numerous investigators have reported animal models with fatal infections following intravenous injection of conidial or yeast suspensions of *S. schenckii* (1, 4, 10, 17). In general, inoculation of $\geq 10^6$ cells per mouse resulted in uniform mortality. Therefore, the mortality results of the present study are consistent with previously published results. The results of the quantitative organ cultures are generally consistent with those reported by Kwon-Chung (11). She found similar exponential rates of growth, as determined by time course cultures of homogenates of testes or cultures of pooled homogenates of livers, lungs, spleens, and kidneys from mice experimentally infected with strains of *S. schenckii* capable of growth at 37°C. In strains capable of growth at 35 but not 37°C, exponential growth in vivo appeared to occur only in the testes (11). In the models described by Miyaji and Nishimura (14, 15), the liver was the most severely affected organ.

The use of an animal model is important in demonstrating the biological activity of groups of organisms that may appear to be morphologically similar or identical. Thus, we used a mouse model of intravenous infection to compare all environmental isolates recovered from the 1988 outbreak of sporotrichosis. Although all of the isolates produced a *Sporothrix* anamorph characterized by conidia arranged sympodially on the inflated apices of lateral conidiophores, only those that produced dematiaceous colonies and sleeves of melanized conidia on PDA and that grew at 37°C produced fatal infections in mice. The results of the animal model supported the mycological identification of *S. schenckii* for the clinical and environmental group I isolates that grew at 37°C. DNA typing studies that are in progress further substantiate that this group of environmental isolates of *S. schenckii* is identical to the clinical isolates from the epidemic (3).

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