

CONCISE COMMUNICATION

Development of a Murine Model of Cerebral Aspergillosis

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Central nervous system (CNS) *Aspergillus* infection has a mortality rate in humans that approaches 95%. Because no animal models are available for studying this infection, we sought to develop a murine model of CNS aspergillosis. Inconsistent data were obtained for non-immunosuppressed CD-1, C57BL/6, and DBA/2N mice after infection by midline intracranial injection of *Aspergillus fumigatus*. CD-1 mice given cyclophosphamide to produce immunosuppression had continuous pancytopenia. Dose-finding studies in CD-1 mice showed that infection with 5×10^6 conidia/mouse consistently caused 100% mortality by day 5–8; no mice died before day 3. Histologic examination of samples of brain tissue showed focal abscesses containing *Aspergillus* hyphae. Fungus burdens in brain were higher than those in other organs, although *Aspergillus* disseminated to the kidneys and the spleen. The model we established provides an opportunity to study immune responses to and therapeutic options for CNS disease in an immunologically defined, genetically manipulable, and inexpensive species.

Infections caused by *Aspergillus* species have become more and more common over the last several decades [1]. This is thought to be the result of increases in the numbers of immunocompromised patients (i.e., patients receiving chemotherapy with cytotoxic agents and patients receiving immunosuppressive therapy in conjunction with bone marrow and solid-organ transplantation or stem cell replacement), as well as to the improved survival of patients with AIDS.

The central nervous system (CNS) is the most common site of hematogenous dissemination of *Aspergillus* from the respiratory tract. The mortality rate associated with CNS aspergillosis in humans, despite the availability of antifungal therapy, approaches 95% [2]. There is clearly a need to evaluate better treatment strategies to improve clinical outcomes for the growing number of patients with such infections.

To this end, we have developed a murine model of CNS aspergillosis. To model this disease in mice, we sought 100% mortality, so that no animal would have insufficient progression of disease and resolution. However, rapid mortality was not desirable, because of the need for sufficient time for evaluation of potentially useful therapeutic interventions. Given these factors, we attempted to develop a model in which 100% mortality would occur over the course of several days.

Materials and Methods

Preparation of the inocula. *Aspergillus fumigatus*, strain AF-10, which was cultured from a patient with pulmonary aspergillosis and stored at the California Institute for Medical Research (San Jose), was used for all experiments. The isolate was incubated on potato dextrose agar (PDA) plates at 35°C for 48 h and allowed to form conidia. The conidia were harvested in 0.05% (vol/vol) Tween 80 in saline and filtered through several layers of sterile gauze to remove large clumps and hyphal fragments. The conidial suspension was stored at 4°C and used within 1 month. Three days before infection, serial dilutions of the conidial suspension were made and placed on PDA plates. The colonies were counted 1 day before infection. The number of colony-forming units in the conidial suspension was determined, and appropriate dilutions were made to achieve the desired inoculum. The final suspension was diluted with 0.05% Tween 80 and saline so that the desired number of conidia would be available in a final volume of 50 μ L.

Mice. Five-week-old male CD-1, C57BL/6, and DBA/2N mice were purchased from Charles River Laboratories. Mice were infected within 3 days of arrival. On the day of infection, CD-1 and C57BL/6 mice had an average weight of 25 g, and DBA/2N mice

Received 20 February 2002; revised 15 April 2002; electronically published 2 August 2002.

Presented in part: 39th annual meeting of the Infectious Diseases Society of America, San Francisco, 25–28 October 2001 (abstract 612).

Experimental procedures were reviewed and approved by the California Institute for Medical Research's Animal Care and Use Committee and were in accordance with the guidelines of the National Institutes of Health.

Financial support: Foundation for Research in Infectious Diseases.

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The Journal of Infectious Diseases 2002; 186:574–7

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 0022-1899/2002/18604-0020\$15.00

had an average weight of 18 g. All animals were housed in groups of 5 mice per cage and provided sterilized food and acidified water ad libitum. Groups consisted of 10 mice in all experiments.

Infection. Mice were anesthetized by inhalation of methoxyflurane vapors. The appropriate number of conidia in a volume of 50 μL of 0.05% Tween 80 and saline were inoculated intracerebrally at a point midline on the cranium, 4–5 mm posterior to the eyes. A 27-gauge disposable needle was used to deliver the inoculum to a depth of 2–3 mm. Mice were fully recovered within 5 min of the procedure, and no deaths resulted from the inoculation procedure.

Immunosuppression. Mice were given cyclophosphamide intraperitoneally (ip) at 200 mg/kg. The first dose was given 2–3 days before the infection. The time between additional doses was evaluated to determine an optimal immunosuppressive regimen. Mice received a minimum of 2 doses in each experiment.

Peripheral white blood cell (WBC) counts. Samples of 100 μL of blood from the tail vein were obtained in subsets of mice at multiple time points after administration of cyclophosphamide. A determination of total WBC count was done by hemacytometer counting to assess the level of immunosuppression. In addition, blood smears were stained with Giemsa stain, and WBC types were determined.

Survival studies. Cages were inspected twice daily. All animals were observed for a total of 15 days after infection. Mice that survived to day 15 were euthanized; CO_2 vapor was used to produce anoxia.

Fungal cultures and histopathologic examination. Mice were euthanized 1 or 3 days after infection with *Aspergillus*. Brain specimens used for histopathologic examination were fixed in 10% neutral buffered formalin and processed for paraffin embedment. Evaluations were done on sections stained with hematoxylin-eosin and periodic acid–Schiff stain. Organs used for fungal culture were removed using a sterile technique and mechanically homogenized in sterile saline with a Tissumizer (Tekmar). Homogenates were further diluted in saline and quantitatively cultured on Sabouraud dextrose agar plates with 50 mg/L of chloramphenicol, and plates were incubated at 35°C for 48 h. Colonies appearing on plates were counted to determine the fungus burdens remaining in the organ. Plates containing no colonies were kept for 5 days before they were discarded.

Statistical analysis. Survival was analyzed with the Mantel-Haenszel log-rank test, using GraphPad Prism software (version 3.00 for Windows). Fungus burden values were converted to \log_{10} colony-forming units, and those data were compared using the Mann-Whitney *U* test.

Results

Inoculation studies in nonimmunosuppressed mice. Non-immunosuppressed CD-1 mice were highly resistant to infection by intracerebral inoculation of *Aspergillus* conidia. Survival among CD-1 mice was >60% with inocula $\leq 10^7$ conidia/mouse. The highest inoculum, 5×10^7 conidia/mouse, produced death in all animals by day 2. In repeated experiments, all inocula proved to be lethal in <50% of the mice or to be lethal to 100% of the mice in the first 2 days. Similar dose-finding studies were performed with 2 inbred strains of mice: C57BL/6 and DBA/2N. The results of the inoculum studies in C57BL/6 were similar

to the results for nonimmunosuppressed CD-1 mice. In repeated experiments, infection produced rapid mortality at high inocula and minimal mortality at all lower inocula. Similar results were found for DBA/2N mice, although these mice were slightly more susceptible to infection (data not shown).

Effect of immunosuppression on CD-1 mice. The total WBC and differential cell counts for CD-1 mice were determined to assess the effects of different regimens of cyclophosphamide. The total peripheral WBC counts for a period of 15 days in uninfected mice that were given cyclophosphamide at 200 mg/kg every 5 days were determined. WBC counts remained below the normal range (6×10^3 – 15×10^3 cells/ mm^3) beginning 2 days after administration of the first dose of cyclophosphamide. Neutrophil counts were <100 cells/ mm^3 within 3 days of administration of the first dose of cyclophosphamide and remained <100 cells/ mm^3 throughout the experimental period when the drug was given every 5 days.

Intracerebral infection of immunosuppressed CD-1 mice. Immunosuppressed CD-1 mice demonstrated a dose response to intracerebral infection with *A. fumigatus* conidia 2 days after the first dose of cyclophosphamide. Figure 1A shows represen-

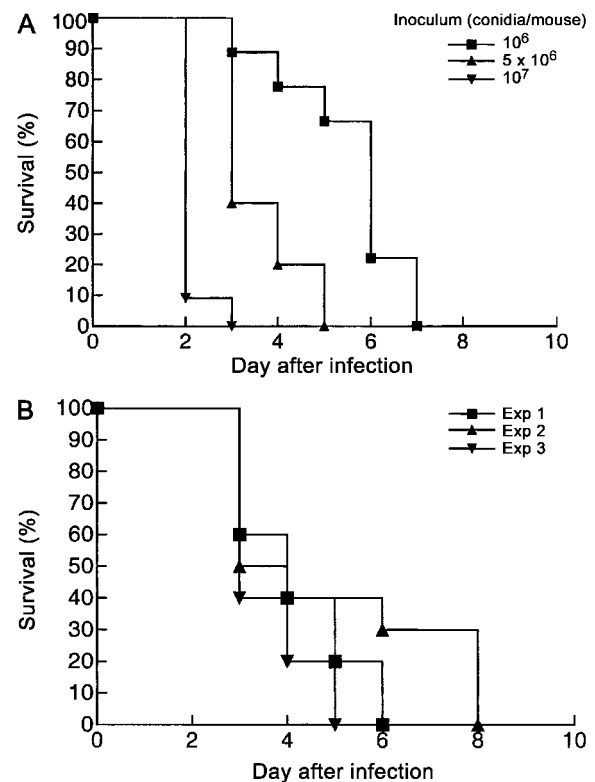


Figure 1. Survival curves for immunosuppressed CD-1 mice that received an injection of intracerebral *Aspergillus fumigatus*. Mice were given cyclophosphamide, 200 mg/kg every 5 days. *A*, Comparison of the survival rates for mice given 3 different intracerebral inocula of *A. fumigatus*. *B*, Survival results from 3 experiments in which an intracerebral inoculum of 5×10^6 conidia of *A. fumigatus* was used.

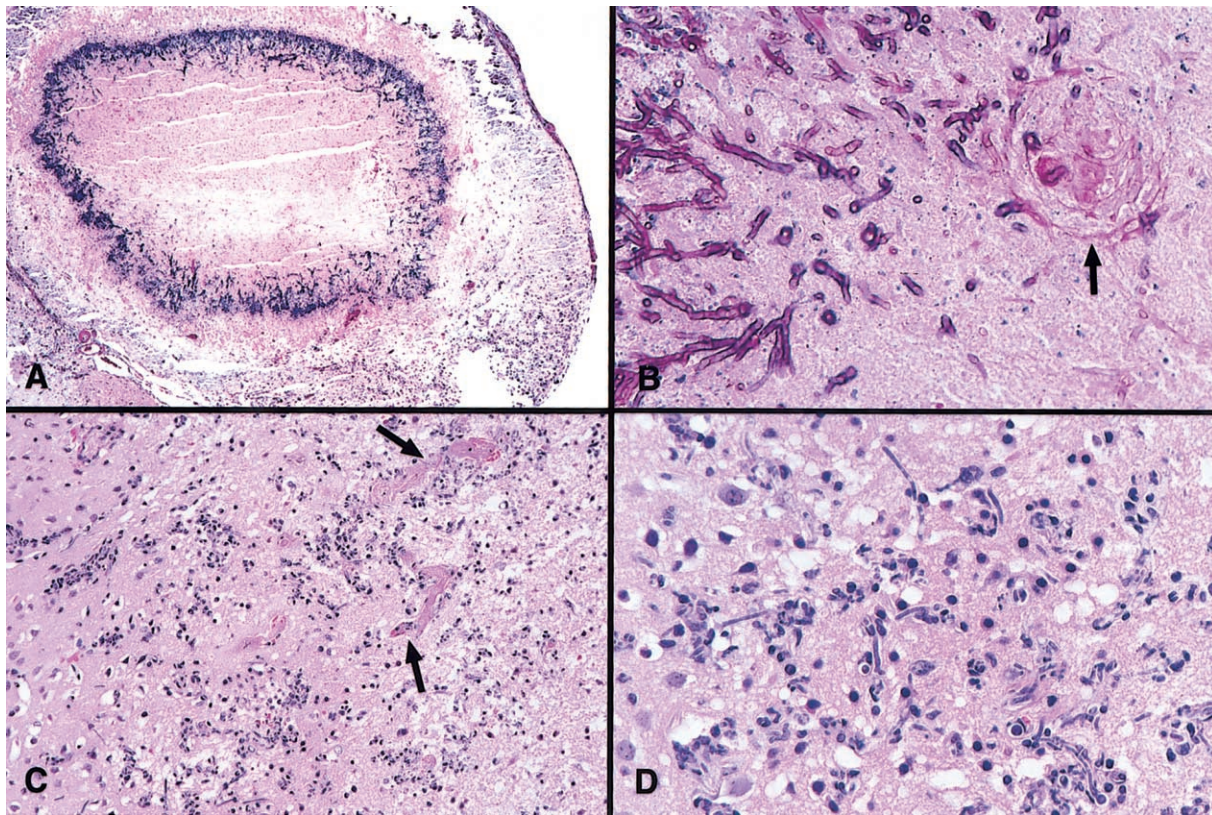


Figure 2. *A*, Abscess in the cerebrum of a CD-1 mouse 3 days after infection with *Aspergillus fumigatus*. Central necrosis with a peripheral rim of *Aspergillus* organisms, surrounded by fibrin and reactive gliosis, can be seen. (Hematoxylin-eosin stain; original magnification, $\times 62$.) *B*, High-power field from the edge of the abscess showing branching septate hyphae and a necrotic thrombosed vessel (arrow). (Periodic acid-Schiff stain; original magnification, $\times 620$.) *C*, Diffuse necrotizing cerebritis in the brain of a CD-1 mouse 3 days after infection. Thrombosed microvessels (arrows), perivascular mononuclear cell infiltrates, and scattered areas of necrosis without formation of a frank abscess are visible. (Hematoxylin-eosin stain; original magnification, $\times 248$.) *D*, Higher-power field showing multiple fungal filaments within capillaries. (Hematoxylin-eosin stain; original magnification, $\times 620$.)

tative data from 3 experiments in which 3 different intracerebral inocula each produced 100% mortality. On the basis of the results of the WBC counts, we chose to use a cyclophosphamide regimen of 200 mg/kg ip, given 2 days before infection and then every 5 days after the first administration of the drug, and an *Aspergillus* inoculum of 5×10^6 conidia/mouse. Figure 1*B* shows the results of 2 additional experiments in which this inoculum and immunosuppressive regimens were used that demonstrate the reproducibility of this model.

Fungus burdens and histopathology. Brains, spleens, and kidneys of immunosuppressed CD-1 mice were removed on day 1 and day 3 after infection to determine fungus burden. The mean fungus burden per brain ($n = 5$ brains) 1 day after infection was $4.4 \log_{10}$ cfu, compared with 3.1 and $3.9 \log_{10}$ cfu for kidney and spleen, respectively. These values were not significantly different. Similarly, 3 days after infection, the mean fungus burden per brain was $4.2 \log_{10}$ cfu, compared with 3.5 and $3.4 \log_{10}$ cfu for kidneys and spleen, respectively.

Histopathologic examination of sections of brain tissue from

mice euthanized on day 3 after infection demonstrated well-organized intracerebral abscesses with central coagulative necrosis, collections of fungal hyphae on the outer perimeter that were surrounded by fibrin, and a glial reaction (figure 2*A* and 2*B*). Thrombosed vessels were seen in the abscess centers. Other areas had a more diffuse pattern of infiltration of the fungal hyphae that was associated with acute necrosis and some perivascular mononuclear cell infiltrates (figure 2*C* and 2*D*).

Discussion

This study reports the development of a murine model of cerebral aspergillosis in neutropenic/pancytopenic outbred mice, the first, to our knowledge, in which mice have been infected intracerebrally. An inoculum of 5×10^6 conidia produced a predictable infection and 100% mortality by day 8 in CD-1 mice that were given cyclophosphamide beginning 2 days before infection and then every 5 days after the first administration of the drug. Results for the nonimmunosuppressed inbred strains

of mice, DBA/2N and C57BL/6, were not consistent, even though these strains have been shown to be more susceptible to a variety of fungal infections, including CNS infection with the fungus *Saccharomyces cerevisiae* and pulmonary infection with *A. fumigatus* [3, 4].

In this model, the infection spreads to other organs, but the fungus burden in the brain remains higher than the burdens in the spleen and the kidney. This differs from other murine models of systemic aspergillosis, in which the fungus may spread to the brain but is often cleared and mice die with very high fungus burdens in the kidneys [5]. Pulmonary aspergillosis in models that require immunosuppression before disease can be produced is rapidly progressive in the lung [4], which eliminates the possibility of studying disseminated disease in a consistent fashion. The model used in the present study produces consistent brain infection with high fungus burdens and focal abscess formation. The use of intracerebral inoculation is associated with few procedure-related deaths and results in a lethal infection in all mice, as demonstrated in the survival studies. The intracerebral route of infection has also been reported in models of both *Cryptococcus neoformans* and *Coccidioides immitis* [6, 7]. Both of these models also produce multiorgan infections and have been used to study the effects of therapy on CNS infection.

This model was developed to provide a means of evaluating therapeutic strategies for CNS infection with *Aspergillus* and of studying the effects of immunomodulation on host defense. Mice are immunologically well-defined, and CD-1 mice are in-

expensive. Other immunosuppressive regimens, other species of *Aspergillus*, and the host response can now be addressed in future studies.

Currently, there are no good treatment options available for patients who develop CNS *Aspergillus* infection. This model should provide a mechanism to study treatments that could be used in clinical trials. Therapeutic strategies that improve survival or lessen pathology and fungus organ burdens can be evaluated using our model.

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