INFECTION AND IMMUNITY, May, 1989, p. 1452–1456
0019-9567/89/051452-05$02.00/0

Vol. 57, No. 5

Fungus Dose-Dependent Primary Pulmonary Aspergillosis in Immunosuppressed Mice

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Received 1 August 1988/Accepted 3 February 1989

We report on a model of primary pulmonary aspergillosis occurring after intranasal instillation of concentrated suspensions of conidia of *Aspergillus fumigatus* in immunocompromised mice. Unconcentrated suspensions of inoculum contained ca. 2×10^7 conidia per ml (1×). These suspensions were concentrated by centrifugation, adjusted to give ca. 2×10^8 (10×) or 2×10^9 (100×) conidia per ml, and delivered in 30-μl droplets to the nares of anesthetized mice. Mice were untreated or injected with cortisone acetate (CA) or cyclophosphamide (CY) in various dosage regimens. It was not possible to obtain mortality of more than 50% with sublethal immunosuppressive treatment and 1× fungus. In contrast, mortality followed a fungus dose response in mice receiving sublethal immunosuppression with either CA or CY. Mortality rates of up to 100% were obtained with 100× fungus and a single dose of CY (200 mg/kg) or CA (250 mg/kg) or three alternate doses (125 mg/kg per day) of CA prior to infection. This model is applicable to the study of acute, fatal primary pulmonary aspergillosis and chemotherapy trials.

There is need for a model of primary pulmonary aspergillosis where mouse mortality is high and dependent upon the dose of fungus inoculated. Basic methods for establishing primary pulmonary infections with the medically important fungi include intranasal instillation of droplets (8, 16), intratracheal injection of suspensions (11, 19), and use of aerosol infection chambers (3, 12–14, 18). The intranasal instillation method is, in principle, simple and expedient. However, our preliminary experiences confirmed what others have reported with this method: the difficulty in achieving consistent and reproducible mortality in mice inoculated intranasally (11).

We tested numerous immunosuppression regimens and various modifications of inoculum preparation in order to develop a model of primary pulmonary aspergillosis that is clinically relevant. The intranasal infection model reported here shows that fungus dose-dependent mortality can be obtained reproducibly in immunosuppressed mice with the appropriate concentration of fungus.

MATERIALS AND METHODS

Mice. Swiss white male mice (Fuellinsdorf, Switzerland) were used and weighed 18 to 20 g at the time of infection. Animals were maintained as described previously (1).

Fungi. Two strains of *Aspergillus fumigatus* were used: DMD 401 from invasive human disease and CM 437 from the Roche Culture Collection. There were no statistically significant differences in the results obtained with the two strains; therefore, the results were combined.

Inoculum. Cultures were grown on potato dextrose agar (Difco Laboratories, Detroit, Mich.) slants at 37°C for five days. Inoculum was prepared by washing surface growth with 0.1% Tween 80 in sterile physiological saline and filtering the resulting suspension through two layers of gauze. This routinely resulted in 2×10^7 conidia per ml (1×, unconcentrated). For concentration of the inoculum, Roux flasks were used to prepare potato dextrose agar slants containing 80 ml of agar per flask. Conidial suspensions were spread over the agar surfaces of the slants, which were incubated as above. Conidia were harvested in 100 ml of sterile physiological saline and centrifuged at 6,800 × *g*. Conidia were suspended in sterile physiological saline to give concentrations of either 2×10^8 (10×) per ml or 2×10^9 (100×) per ml. Cell numbers were determined in a hemocytometer. Conidial viability was greater than 99% as determined by dilution pour plates.

Intranasal inoculation. Mice were anesthetized with a mixture of absolute ethanol-chloroform-ether (1:2:3) as described by Waldorf (A. R. Waldorf, Ph.D. dissertation, University of California, Berkeley, 1981). Anesthesia times in a typical experiment were 74 ± 9 s. During inoculation, mice were held perpendicular to the counter top and the mouths were pressed shut. Inoculum was delivered to the nares in a single droplet of 0.03 ml from a micropipette. Mice inhaled the droplet involuntarily. Noses were decontaminated with 70% ethanol, and mice were kept in plastic cages which were fitted with cardboard filter tops. Animals were observed twice each day over a 20-day period, and mortality was recorded.

Immunosuppression. Cortisone acetate (CA; Sigma Chemical Co., St. Louis, Mo.) was suspended in sterile water containing 0.1% Tween 80. Doses were either 125 or 250 mg/kg injected subcutaneously in 0.5 ml or less. The high dose was given on the day before infection. The low dose was given on three alternate days per week (beginning on Monday with infection on Friday) for the three-dose regimen. Alternate-day dosing was continued for two additional weeks for the nine-dose regimen. Cyclophosphamide (CY) was purchased as the injectable form and given intravenously at 200 mg/kg 2 days before infection (Endoxan; Astea Co., Bonn, Federal Republic of Germany).

Quantitative lung cultures. Animals were killed immediately after inoculation. Lungs were removed and homogenized separately in sterile physiological saline in sterile glass tissue grinders. CFU were determined by using serial dilu-

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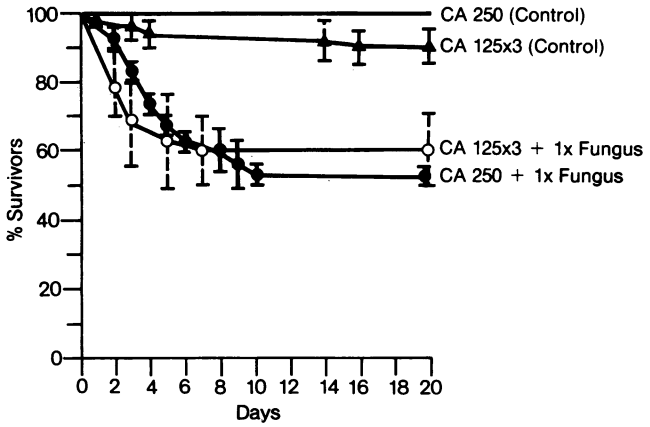

FIG. 1. Standard inoculum of *A. fumigatus* does not result in uniformly fatal disease in mice given sublethal immunosuppression with CA and intranasal challenge. Drug doses are in milligrams per kilogram; 1× fungus, 6×10^5 conidia per mouse. Bars, Mean ± standard error.

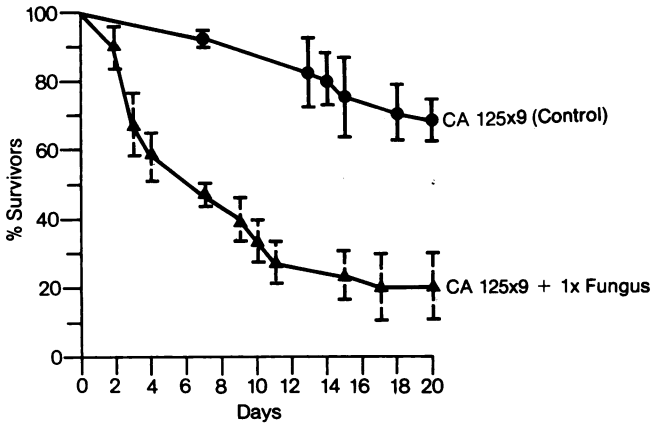

FIG. 2. Increase in the duration of CA immunosuppression results in increased mortality in both uninfected controls and mice infected intranasally with *A. fumigatus*. Drug doses are in milligrams per kilogram; 1× fungus, 6×10^5 conidia per mouse. Bars, Mean ± standard error.

tions and Sabouraud agar (Difco) pour plates supplemented with gentamicin (10 mg per liter) and chloramphenicol (20 mg per liter).

Statistics. All experiments were repeated from two to nine times (mean of three), and the results were pooled. Groups of 5 to 10 mice were used per infection dose. In each experiment, 10 animals were used as controls for the immunosuppression procedure (no infection); 10 animals were used as controls for the infection procedure in normal mice (no immunosuppression). Survival times of infected mice were compared with those of immunosuppressed controls and tested individually in each experiment. Different fungus dose groups of mice were compared within an individual experiment and then the results of repeated experiments were pooled. Pooled data were expressed. When the data from the pooled experiments were significant, the data from the individual experiments were also significant.

Survival times were analyzed by the Wilcoxon rank sum test. Animals that survived had the highest rank. The animals that died earliest had the lowest rank. CFU data (as logarithms) were analyzed by the Student *t* test.

RESULTS

Control mice with no immunosuppression did not die after intranasal inoculation of *A. fumigatus* at any of the concentrations used in this study (up to 6×10^7 conidia per mouse). In contrast, mortality did result in mice immunosuppressed with either CA or CY and infected with *A. fumigatus*.

CA at 250 mg/kg given once or 125 mg/kg given three times resulted in significant increases in mortality after infection with *A. fumigatus*, compared with the uninfected, immunosuppressed controls ($P \leq 0.05$; Fig. 1). There was no significant difference between the two methods of immunosuppression. Most important, mortality was not greater than 50% under these conditions with the unconcentrated inoculum.

Extending the CA dosage of 125 mg/kg to nine alternate weekdays over a 3-week period resulted in increased morbidity in both uninfected and infected animals. The degree of dehydration and weight loss due to CA as judged in the immunosuppressed control groups was severe. There was a trend for mortality to be increased in both the uninfected and the infected animals receiving the additional doses of cortisone (Fig. 2), compared with animals receiving only three doses of the drug (Fig. 1). These differences were not statistically significant. However, the difference between mortality in the uninfected animals and that in the infected animals receiving nine doses of CA at 125 mg/kg was significant ($P < 0.01$). Yet, mortality in the fungus infection groups never reached 100%.

In contrast, increasing the concentration of fungus by either 10- or 100-fold to 10^8 or 10^9 conidia per ml, respectively, had dramatic effects upon mortality in this model with either regimen of CA immunosuppression (Fig. 3 and 4). With three alternate-day CA doses of 125 mg/kg, either the 10× or 100× inoculum resulted in significant increases in mortality over uninfected controls ($P < 0.01$ for both groups; Fig. 3). With the 100× inoculum, 100% mortality was consistently achieved. Although there was more mortality with the 100× inoculum than with the 10× inoculum, the comparative difference was of borderline significance ($P \geq 0.1$). With a single CA dose of 250 mg/kg, either the 10× or 100× inoculum also resulted in significant increases in mor-

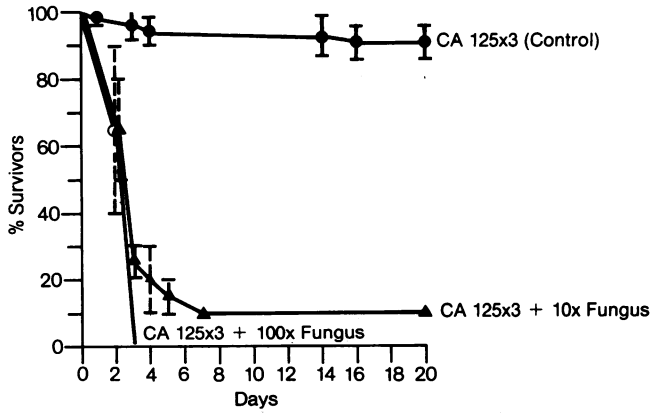

FIG. 3. Mouse mortality in a primary pulmonary model of aspergillosis is fungus dose dependent in mice immunosuppressed with sublethal CA given in three alternate-day doses. Drug doses are in milligrams per kilogram; 10× fungus, 6×10^6 conidia per mouse; 100× fungus, 6×10^7 conidia per mouse. Bars, Mean ± standard error.

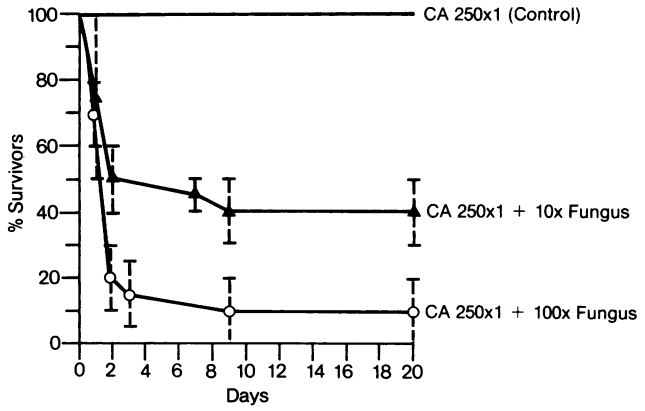

FIG. 4. Mouse mortality in a primary pulmonary model of aspergillosis is fungus dose dependent in mice immunosuppressed with sublethal CA given in single doses. Drug doses are in milligrams per kilogram. 10× Fungus, 6×10^6 conidia per mouse; 100× fungus, 6×10^7 conidia per mouse. Bars, Mean ± standard error.

tality over uninfected controls ($P < 0.05$ and $P < 0.01$, respectively; Fig. 4). Again, the difference between the 10× and the 100× inoculum was of borderline significance ($P < 0.02$ from one trial; $P > 0.01$ from a second trial). Thus, with either regimen of CA immunosuppression, there was a general mortality response to fungus dose.

This same pattern of increased mortality in response to increased concentration of fungus was seen in the mice immunosuppressed with CY (single dose of 200 mg/kg) and inoculated with 100× fungus (Fig. 5). Significant increases ($P < 0.01$) in mortality of up to 100% were seen under these conditions. With this means of immunosuppression, there was no significant increase in mortality after inoculation with the 10× inoculum of fungus.

More than half of the inoculum (mean of 56% from three groups of 10 mice each; independent experiments) was cultured from the lungs immediately after intranasal challenge of normal mice (Table 1). More fungus was recovered from the lungs of normal mice than from cortisone-treated mice, but the difference was not statistically significant. In contrast, there was a statistically significant increase in the

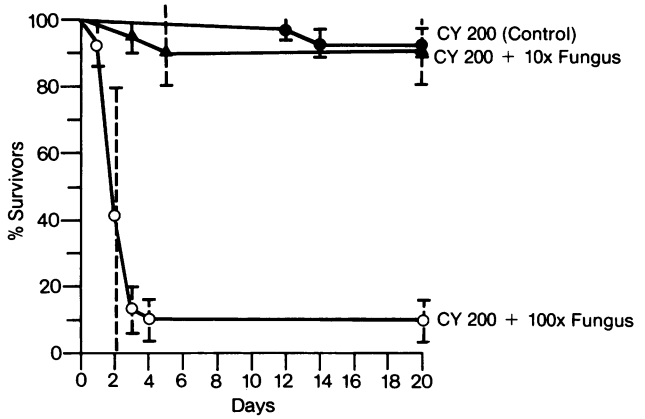

FIG. 5. Concentration of *A. fumigatus* by 100-fold produces fatal disease in mice immunosuppressed with single doses of CY. Drug doses are in milligrams per kilogram; 10× fungus, 6×10^6 conidia per mouse; 100× fungus, 6×10^7 conidia per mouse. Bars, Mean ± standard error.

TABLE 1. Recovery of *A. fumigatus* from the lungs of mice immediately after intranasal inoculation[a]

Group of mice (inoculum)[b]	Log CFU delivered per mouse	Log CFU recovered per lung ± SE
Cortisone treated (1×)	5.7076	5.5993 ± 0.1193
Normal (1×)	5.7076	5.5551 ± 0.0731
Normal (10×)	6.8195	6.5469 ± 0.1910
Normal (100×)	7.8195	8.0183 ± 0.2685

[a] Results of a typical experiment with groups of 10 mice. Results were reproducible.

[b] Statistical significance among groups with or without concentrated inocula: cortisone-treated 1× versus normal 1×, not significant ($P > 0.50$); normal 1× versus normal 10×, significant ($P < 0.001$); normal 10× versus normal 100×, significant ($P > 0.001$); normal 1× versus normal 100×, significant ($P < 0.001$). Treated mice were given 250 mg of cortisone per kg once.

amount of fungus recovered from the lungs of normal mice receiving inoculum concentrated 10- or 100-fold, compared with the amount recovered from normal mice receiving unconcentrated inoculum ($P < 0.001$). Also, there was a statistically significant increase in CFU recovered from the lungs of mice receiving the 10× versus the 100× inoculum concentrations ($P < 0.001$).

DISCUSSION

We initally thought our inability to establish uniformly fatal infections with *A. fumigatus* in immunosuppressed mice was related to either technical difficulties with the model or inappropriate immunosuppression of the mice. Numerous repetitions ruled out the former, and numerous trials of various immunosuppression regimens ruled out the latter. We have shown here that various modifications of CA and cyclophosphamide immunosuppression failed to yield uniformly fatal disease in mice challenged intranasally with unconcentrated *A. fumigatus*. In addition to the modifications we report here, we have also tested consecutive versus alternate daily doses of CA, different formulations of CA, different types of corticosteroid (hydrocortisone), multiple doses of CY, and different routes of administration of CY, all with essentially unrewarding results.

In contrast, there was a dramatic increase in mouse mortality after modifications were made in the dose of fungus given. Concentration of the inoculum 100-fold gave 100% mortality with either CA or CY immunosuppression. These increases in mortality over values from mice receiving unconcentrated inoculum were statistically significant. A 10-fold concentration of the inoculum also resulted in increased mortality rates relative to the unconcentrated inoculum groups. For the mice receiving the three-dose (125 mg/kg per dose) regimen of CA immunosuppression, these differences were also significant. For mice receiving the single CA dose of 250 mg/kg, there was only a 10% increase in cumulative mortality at day 20; however, there was a definite increase in the initial rate of mortality as seen from the 50% mortality at day 2, compared with 5% mortality in the group receiving unconcentrated inoculum.

Other workers have demonstrated the importance of fungus dose dependency in the intranasal infection model with opportunistic fungal pathogens including *A. fumigatus*. Waldorf et al. (17) found dose-dependent increases in the CFU of *Rhizomucor pusillus* recovered from the lungs of Fuellinsdorf mice immunosuppressed with a single dose of 250 mg of CA per kg. Approximately 10% of the inoculum was recovered from the lungs at each challenge concentration, and

infection was fungus dose dependent. In another study, Waldorf et al. (16) reported a 50% lethal dose of 1.5×10^2 conidia of *A. fumigatus* in CD-1 mice immunosuppressed with six consecutive daily doses of 125 mg of CA per kg and infected intranasally. In this system, 1.5×10^6 conidia resulted in 80% mortality. However, since the objectives of their study were somewhat different, dose-response curves were not given.

Graybill and co-workers have also reported intranasal infection studies with *A. fumigatus* in mice (5–7). Similar to our findings, immunosuppression was necessary to establish fatal infections. After mortality was noted in uninfected control mice receiving five cortisone doses of 2.5 mg per mouse per day, the number of doses was reduced to three. They obtained 90 to 100% mortality with two different challenge doses of separate isolates of fungus. These investigators also were able to demonstrate a dose effect.

Sidransky and Friedman used an aerosol infection chamber for infecting mice with *Aspergillus flavus* (15). Mortality of 67% was obtained in mice receiving a single 5-mg dose of CA 2 days before inoculation. The administration of antibacterial antibiotics to the mice appeared to increase maximum mortality rates to 88%. There appeared to be a fungus dose-dependent response with mortality rates of 30, 50, and 88% after exposure to low, medium, and high doses of spore exposure. It is not possible to determine with certainty how the doses of fungal spores were altered, but this may have been done by varying the exposure time. Thus, these results, obtained with an aerosol infection chamber, are in general agreement with our data derived from intranasal inoculation of mice immunosuppressed with a single (250 mg/kg) dose of cortisone. Hence, the aerosol infection chamber yielded results consistent with ours from intranasal inoculation. However, the chamber in the preceding study resulted in whole body contamination of the animals and would appear to be difficult to control for uniform delivery of a predetermined inoculum to the mice.

Sidransky and colleagues as well as other workers have used aerosol infection chambers of the sort described by Piggot and Emmons (12) for efficiently establishing experimental aspergillosis (3, 13, 18). The Piggot and Emmons chamber eliminates the whole body exposure of the animals to spores, since only the noses extend into the central part of the flask. Yet, it is slower to use than the bell jar exposure chamber described in the original work of Sidransky and Friedman (15) because it is difficult to place the mice in the sidearm tubes extending into the flask.

More recently, Schaffner et al. (14) used such a chamber fitted with manifold tubes to infect mice with conidia of *A. fumigatus*. Various immunosuppression regimens were used, including CA. With a single injection of 250 mg of CA per kg, there was a fungus dose response associated with mortality. Values ranged from 0 to 89% mortality over a fungus concentration range of 2.5×10^5 to 8×10^6 conidia per mouse as determined from CFU recovered from lungs. Thus, these data are consistent with our own with the intranasal inoculation method. In fact, we have used the methods of Schaffner et al. to give mortality of 100% in mice immunosuppressed with three alternate-day doses of 125 mg of CA per kg. Therefore, this method meets our criteria of establishing a primary pulmonary infection with uniform mortality. Furthermore, it represents a true aerosol infection, and it is less artificial than the droplet instillation method used in the present study. However, we find the method to be very slow, it is too difficult to control prospectively the concentration of fungal inoculum, and the method is not easily adapted to the uniform infection of large numbers of animals as is required in chemotherapy studies. The method also causes considerable surface contamination of the mouse noses. These problems are not encountered with the intranasal infection model.

We used normal mice for determining inoculation efficiency because this obviated the need of handling the mice from one to nine additional times for dosing with the immunosuppressive drug. We were initially concerned that there may be differences in the inoculation efficiency in the immunosuppressed mice as was discussed in another model of intranasal infection with a different fungus (2). However, preliminary studies indicated that the number of *A. fumigatus* CFU were not statistically different from the two groups of mice.

One goal of these studies was to establish a model of primary pulmonary aspergillosis for the evaluation of antifungal agents. A standard of comparison for treatment in this disease is amphotericin B. A toxic interaction between amphotericin B and CA in adult female CD-1 mice was observed by Kisch et al. (10). Dose-related mortality and renal tubular epithelial lesions occurred within six days of treatment. Graybill et al. later reported that intranasally challenged BALB/c mice treated with corticosteroids and amphotericin B had increased mortality which precluded evaluation of amphotericin B in this animal model (5–7). The mechanism of this synergistic toxicity has not been elucidated. One possible mechanism may be a combination of the toxic effect on amphotericin B on renal tubular epithelial cells and the glucocorticosteroid-induced impairment of reparative protein synthesis required for tubular epithelial regeneration. Another possible mechanism may be that of hypokalemia or intracellular potassium depletion. Amphotericin B is well known to cause hypokalemia and kaliuresis (4). Glucocorticosteroids also cause hypokalemia and have been found to be more potent kaliuretics than aldosterone (9). The renal tubular epithelial lesions of hypokalemia histopathologically reveal cytoplasmic swelling similar to that of the lesions described by Kisch et al. (10). Further investigations, including metabolic studies on the combined renal effects of amphotericin B and glucocorticosteroids, are warranted to clarify these possible mechanisms.

We have not evaluated the combination of amphotericin B and CA in the intranasally inoculated mice in our experiments. If synergistic toxicity becomes a problem in such animals receiving amphotericin B or other antifungal drugs, then the cyclophosphamide-immunosuppressed mice may be utilized. Alternatively, perhaps the tolerance of CA-immunosuppressed mice to amphotericin B may be improved by other modalities. For example, increased dietary sodium may reduce amphotericin B nephrotoxicity by suppression of the tubuloglomerular feedback. Increased dietary potassium may possibly spare development of hypokalemic nephrotoxicity.

We summarize our findings with the intranasal infection model with the following conclusions. (i) Mortality in immunosuppressed mice is dependent upon the amount of *A. fumigatus* inoculated. (ii) Uniform mortality of 100% requires delivery of 10^7 to 10^8 conidia to the lungs. (iii) This requires concentration of a standard (unconcentrated) inoculum up to 100-fold. (iv) Various sublethal immunosuppression regimens are satisfactory. (v) This model is suited to chemotherapy studies in primary pulmonary aspergillosis.

ACKNOWLEDGMENT

We thank Andreas Schaffner for the use of his aerosol infection chamber.

LITERATURE CITED

1. **Dixon, D. M.** 1987. In vivo models: evaluating antifungal agents. Methods Find. Exp. Clin. Pharmacol. **9**:729–738.
2. **Dixon, D. M., W. G. Merz, H. L. Elliott, and S. Macleay.** 1987. Experimental central nervous system phaeohyphomycosis following intranasal inoculation of *Xylohypha bantiana* in cortisone-treated mice. Mycopathologia **100**:145–153.
3. **Epstein, S. M., E. Verney, T. D. Miale, and H. Sidransky.** 1967. Studies on the pathogenesis of experimental pulmonary aspergillosis. Am. J. Pathol. **51**:769–788.
4. **Gouge, T. H., and V. T. Andriole.** 1971. An experimental model of amphotericin B nephrotoxicity with renal tubular acidosis. J. Lab. Clin. Med. **78**:713–724.
5. **Graybill, J. R., and J. Ahrens.** 1985. Itraconazole treatment of murine aspergillosis. J. Med. Vet. Mycol. **23**:219–223.
6. **Graybill, J. R., and S. R. Kaster.** 1984. Experimental murine aspergillosis. Comparison of amphotericin B and a new polyene drug, SCH 28191. Am. Rev. Respir. Dis. **129**:292–295.
7. **Graybill, J. R., S. R. Kaster, and D. J. Drutz.** 1983. Treatment of experimental murine aspergillosis with BAY n7133. J. Infect. Dis. **148**:898–906.
8. **Harvey, R. P., E. S. Schmidt, C. C. Carrington, and D. A. Stevens.** 1978. Mouse model of pulmonary blastomycosis: utility, simplicity, and quantitative parameters. Am. Rev. Respir. Dis. **117**:695–703.
9. **Hiatt, E., L. W. Chapman, M. B. Davidson, and J. A. Sheinkopf.** 1979. Adrenal hormones and the regulation of serum potassium in potassium-loaded adrenalectomized dogs. Endocrinology **105**:215–219.
10. **Kisch, A. L., R. P. Maydew, and A. P. Evan.** 1978. Synergistic nephrotoxicity of amphotericin B and cortisone acetate in mice. J. Infect. Dis. **137**:789–794.
11. **Lawrence, R. M., A. C. Huston, and P. D. Hoeprich.** 1977. Reproducible method for induction of pulmonary coccidioidomycosis in mice. J. Infect. Dis. **135**:117–119.
12. **Piggott, W. R., and C. W. Emmons.** 1960. Device for inhalation exposure of animals to spores. Proc. Soc. Exp. Biol. Med. **103**:805–806.
13. **Richard, J. L., and J. R. Thurston.** 1983. Rapid hematogenous dissemination of *Aspergillus fumigatus* and *A. flavus* spores in turkey poults following aerosol exposure. Avian Dis. **27**:1025–1033.
14. **Schaffner, A., H. Douglas, and A. Braude.** 1982. Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to *Aspergillus*. Observations on these two lines of defense *in vivo* and *in vitro* with human and mouse phagocytes. J. Clin. Invest. **69**:617–631.
15. **Sidransky, H., and L. Friedman.** 1959. The effect of cortisone and antibiotic agents on experimental pulmonary aspergillosis. Am. J. Pathol. **35**:169–183.
16. **Waldorf, A. R., S. M. Levitz, and R. D. Diamond.** 1984. In vivo bronchoalveolar macrophage defense against *Rhizopus oryzae* and *Aspergillus fumigatus*. J. Infect. Dis. **150**:752–760.
17. **Waldorf, A. R., L. Peter, and A. Polak.** 1984. Mucormycotic infection in mice following prolonged incubation of spores *in vivo* and the role of spore agglutinating antibodies on spore germination. J. Med. Vet. Mycol. **22**:101–108.
18. **White, L. O.** 1977. Germination of *Aspergillus fumigatus* conidia in the lungs of normal and cortisone-treated mice. Sabouraudia **15**:37–41.
19. **Williams, D. M., M. H. Weiner, and D. J. Drutz.** 1981. Immunologic studies of disseminated infection with *Aspergillus fumigatus* in the nude mouse. J. Infect. Dis. **143**:726–733.