Pathogenesis of Pulmonary Aspergillosis

Granulocytopenia versus Cyclosporine and Methylprednisolone-Induced Immunosuppression

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Patients with chemotherapy-induced granulocytopenia for neoplastic diseases and those receiving cyclosporin A plus corticosteroids for prevention and treatment of organ transplant rejection are two immunologically distinct patient populations with high risks for development of invasive pulmonary aspergillosis. In order to compare the pathogenesis of aspergillosis in these two high-risk populations and to further characterize the role of cyclosporin A in development of pulmonary aspergillosis, we studied the patterns of infection and inflammation in two clinically applicable rabbit models of invasive pulmonary aspergillosis. There were striking differences in the patterns of infection and inflammation of invasive pulmonary aspergillosis according to the type of underlying immune defect. Among rabbits challenged with the same intratracheal inoculum, there was a 100% mortality for invasive pulmonary aspergillosis in profoundly granulocytopenic rabbits in comparison with a 100% survival in rabbits immunosuppressed with cyclosporin A plus methylprednisolone (CsA+MP). Lesions of pulmonary aspergillosis in granulocytopenic rabbits consisted predominantly of coagulative necrosis, intraalveolar hemorrhage, and scant mononuclear inflammatory infiltrate. By comparison, pulmonary foci in rabbits immunosuppressed by CsA+MP consisted mainly of neutrophilic and monocytic infiltrates, inflammatory necrosis, and scant intraalveolar hemorrhage. There was extensive infiltration by hyphae with angioinvasion in granulocytopenic rabbits, whereas conidia in various stages of germination predominated in CsA+MP-treated animals in which there was a paucity of hyphae or angioinvasion. Extrapulmonary disease predominated in granulocytopenic rabbits. Methylprednisolone was the major immunosuppressive drug in rabbits treated with CsA+MP. Cyclosporin A alone did not increase the progression of pulmonary aspergillosis and did so only when used chronically with methylprednisolone. Berenguer J, Allende MC, Lee JW, Garrett K, Lyman C, Ali NM, Bacher J, Pizzo PA, Walsh TJ. Pathogenesis of pulmonary aspergillosis: granulocytopenia versus cyclosporine and methylprednisolone-induced immunosuppression. AM J RESPIR CRIT CARE MED 1995;152:1079–86.

Invasive pulmonary aspergillosis is an important cause of infectious morbidity and mortality in immunocompromised patients, particularly in those receiving myelotoxic chemotherapy (1–10) and in those undergoing pharmacologic immunosuppression with corticosteroids and cyclosporin A (CsA) for prevention or treatment of rejection after allograft tissue transplantation (11–18). Patients with cancer and transplant recipients are afflicted by different defects in host-defense mechanisms that increase their risk for invasive aspergillosis. Chemotherapy-induced granulocytopenia is the principal defect in patients with cancer, whereas T-cell and phagocyte dysfunction induced by immunosuppressive drugs is characteristic of transplant recipients. Macrophages inhibit the germination of *Aspergillus* conidia and corticosteroids impair this function (19–21).

Little is known, however, about the effect of CsA as a risk factor for invasive pulmonary aspergillosis. Moreover, the effects of profound granulocytopenia versus CsA-corticosteroid immunosuppression on the patterns of infection and inflammation in invasive pulmonary aspergillosis are not well understood. We therefore studied the effects of profound granulocytopenia, methylprednisolone (MP), CsA, and the combination of CsA plus MP (CsA+MP) in rabbit models of invasive pulmonary aspergillosis in order to develop a better understanding of the pathogenesis and patterns of infection in both granulocytopenic patients and patients with pharmacologic suppression of cellular immunity.

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METHODS

Animals

Female New Zealand white rabbits (Hazleton Research Products, Denver, PA) weighing 2 to 3 kg were used in all experiments. They were housed and maintained according to National Institutes of Health guidelines for animal care and in fulfillment of American Association for Accreditation of Laboratory Animal Care criteria. Vascular access was established in each rabbit by the surgical placement of a subcutaneous silastic central venous catheter (22).

Drugs

Cytosine arabinoside (AraC) (Cytosar-U; Upjohn, Kalamazoo, MI) was dissolved with normal saline at 50 mg/ml. Methylprednisolone sodium succinate (Solu-Medrol; Upjohn) was dissolved with normal saline to concentrations of 6.25 or 3.57 mg/ml, stored at room temperature, and used within 48 h. Cyclosporin A in polyoxyethylated castor oil (Sandimmune; Sandoz Pharmaceuticals Corp., East Hanover, NJ) was diluted to 2.5 mg/ml in normal saline, stored protected from light at 4° C, and used within 24 h.

Fungus and Inoculation

A well-characterized strain of *Aspergillus fumigatus* (isolate no. 4215) obtained from a fatal case of pulmonary aspergillosis was used in all the experiments. To detect any possible antifungal effect of CsA (23, 24) on *A. fumigatus*, we used a broth macrodilution method for determination of *in vitro* susceptibility. The inoculum was 1 to 5 $\times$ 10^4 colony-forming units (CFU) per ml in RPMI 1640 medium buffered with 0.165 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) containing L-glutamine and lacking sodium bicarbonate (BioWhittaker, Inc., Walkersville, MD). Tubes were read with 48 h of incubation at 30° C. With this method, concentrations of CsA as great as 160 μg/ml had no effect on growth of this strain of *A. fumigatus*.

The inoculum of *A. fumigatus* for *in vivo* studies was prepared from a frozen isolate that was subcultured onto potato dextrose agar slants, which were incubated for 24 h at 37° C and then maintained at room temperature for 5 d. Conidia were harvested under a laminar airflow hood with a solution of 0.025% Tween 20 (Fisher Scientific, Fair Lawn, NJ) in normal saline, transferred to a 50-ml conical tube, and counted in a hemacytometer. The concentration was adjusted in order to give each rabbit a predetermined inoculum of 2.8 $\times$ 10^8 conidia of *A. fumigatus* in a volume of 200 to 350 μl. The concentrations of inocula were confirmed by serial dilution and culture on Sabouraud glucose agar (SGA) checkplates. Rabbits were inoculated intravenously on Day 2 under general anesthesia with 0.5 to 1.0 ml of a 2:1 mixture (vol/vol) of ketamine 100 mg/ml (Fort Dodge Labs, Fort Dodge, IA) and xylazine 20 mg/ml (Mobay Corp., Shawnee, KA). Once satisfactory anesthesia was reached, a Flagg 0 straight blade laryngoscope (Welch-Allyn, Skaneateles Falls, NY) was inserted until the vocal cords were clearly visualized, and the *A. fumigatus* inoculum was given intratracheally with a tuberculin syringe attached to a 5.25-inch Teflon catheter (Becton Dickinson, Sandy, UT).

Immunosuppressive Regimens

The effects of the following clinically applicable immunosuppressive regimens on invasive pulmonary aspergillosis were studied.

1. *AraC.* Rabbits in these groups received daily doses intravenously of AraC 525 mg/m^2 on Days 1 through 5, and 484 mg/m^2 on Days 8 and 9 to produced profound and persistent granulocytopenia (Granulocyte counts < 500/μl on Day 5, and < 100/μl from Day 6 onward). Supportive treatment included administration of vancomycin in drinking water (50 μg/ml) starting on Day 1 for prevention of antiobiotic-associated diarrhea. Parenteral antibiotics starting on Day 4 for prevention of opportunistic bacterial infections consisted of ceftazidime (Glaxo, Research Triangle Park, NC) 75 mg/kg given intravenously twice daily, gentamicin (Gensia Pharmaceuticals, San Diego, CA) 5 mg/kg given intravenously once every other day, and vancomycin (Eli Lilly, Indianapolis, IN) 15 mg/kg intravenously. This level of profound granulocytopenia developed concomitantly with thrombocytopenia, ranging in most rabbits between 10,000 and 25,000/μl.

2. *Cyclosporin A.* Rabbits were immunosuppressed intravenously exclusively with CsA at doses of 10 or 20 mg/kg/d. The infusion rate of CsA was 0.5 mg/kg each 15 s. A mild acute reaction characterized by cutaneous warmth and generalized erythema developed during the infusion of CsA. These effects abated after the third dose and disappeared by the seventh day of the experiment. The results from immunosuppression at 10 and 20 mg/kg/d were similar and therefore combined. The dosage of 30 mg/kg was associated with unacceptable neurotoxicity and therefore not studied further.

3. *Methylprednisolone.* Rabbits in this group were immunosuppressed with MP, 5 mg/kg/d IV during the first 3 days of the experiment and 2 mg/kg/d IV thereafter.

4. *Cyclosporin A plus Methylprednisolone.* These rabbits were immunosuppressed with CsA (10 or 20 mg/kg/d) in combination with MP 5 mg/kg/d IV during the first 3 days of the experiment and 2 mg/kg/d IV thereafter.

5. *Normal Control.* This group was composed of normal saline-treated nonimmunosuppressed infected rabbits.

In order to control for exogenous infection from environmental sources during immunosuppression, we concluded preliminary studies of immunosuppressed rabbits without *Aspergillus* inoculation. Noninoculated animals were treated with AraC (only), CsA (only), MP (only), and CsA plus MP for 12 to 28 d, depending upon the group. None of the rabbits in these studies had microbiologic evidence of *A. fumigatus* recovered from lung cultures.

Duration of Immunosuppression

The effects of duration of immunosuppression for 12 versus 28 d on the differences in patterns of invasive pulmonary aspergillosis and survival were studied in four different groups: CsA (only) 10 mg/kg; MP (only) 5 mg/kg/d given alone intravenously during the first 3 d of the experiment and 2 mg/kg/d intravenously thereafter; CsA + MP in combination; and saline-treated control.

Outcome Variables

All the experiments were evaluated according to the following outcome variables.

1. *Survival.* Duration of survival in days postinoculation was recorded for each rabbit. Surviving rabbits were killed by pentobarbital anesthesia on Day 11 postinoculation in the model of acute pulmonary aspergillosis and on Day 28 of the chronic pulmonary aspergillosis. Two models were utilized in these studies: acute (12 d) and chronic pulmonary aspergillosis (28 d). Both models received the same inoculum. Because of the high mortality, granulocytopenic rabbits were studied only as an acute model. However, the nongranulocytopenic rabbits, which had a high level of survival, were studied as both acute and chronic models. The study of nongranulocytopenic chronic models permitted investigation of the effect of immunosuppression on pulmonary clearance of *A. fumigatus* over extended time.

2. *Pulmonary Lesion Scores.* The entire heart-lung block was carefully dissected and removed at autopsy and the heart was dissected away from the lungs, leaving an intact tracheobronchial tree and lung preparation. The lungs were weighed (Mettler Instrument Co., Hightstown, NJ) and inspected at least by two observers (blinded to the treatment group) who recorded the type of lesions (if any) in each separate lobe.

3. *Fungal Cultures.* Bronchoalveolar lavage (BAL) was performed on each lung preparation by the instillation and subsequent withdrawal of 10 ml of sterile normal saline three times into a clamped trachea. A 0.1-ml sample of this fluid was cultured on SGA. A representative region of each lobe was subsequently excised for cultures and histologic examination. Each fragment was weighed individually, minced with sterile scissors, and homogenized with sterile saline for 1 min per tissue sample (Stomacher 80; Tekmar, Cincinnati, OH). Lung homogenates in 10^{-2} and 10^{-4} dilutions were prepared in sterile saline, and aliquots of 100 μl were plated onto SGA and incubated at 37° C for the first 24 h and then at room temperature for another 24 h. After this, the CFU of *A. fumigatus* were counted and recorded for each lobe and the CFU/g were calculated. A finding of one colony of *A. fumigatus* was considered positive. In addition, the percentage of culture-positive lobes was calculated for each rabbit.

In order to determine the frequency and extent of extrapulmonary invasive aspergillosis, the kidneys, liver, spleen, and brain were inspected for lesions and cultured for the presence *A. fumigatus*. Tissues with evidence of lesions were sectioned and studied histopathologically.

4. *Histopathology.* Pulmonary and extrapulmonary lesions were excised, sectioned, and preserved in 10% neutral buffered formalin. Fixed specimens were then embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E), periodic acid-Schiff (PAS), and Gomori methenamine silver (GMS) stains. Three types of pulmonary lesions were found at postmortem examination: (*1*) hemorrhagic infarcts (dark red consolidated lesions that corresponded histologically to coagulative necrosis and intraalveolar hemorrhage), (*2*) neutrophilic lesions (firm, aggregated, or confluent grayish-white nodular lesions that corresponded histologically to foci of neutrophilic infiltrates with necrosis), and (*3*) monocytic lesions (firm, confluent, flattened grayish-red lesions with a translucent quality that corresponded histologically to zones of chronic mononuclear inflammation without necrosis).

Statistical Analysis

Comparisons between proportions were done by χ^2 or Fisher's exact test, as appropriate. Comparisons of numerical variables between multiple independent groups were performed by one-way analysis of variance followed by the Student-Newman-Keuls procedure or Kruskall-Wallis procedure followed by the Dunn test, as appropriate. Survival curves were estimated by the Kaplan-Meier product limit method (25).

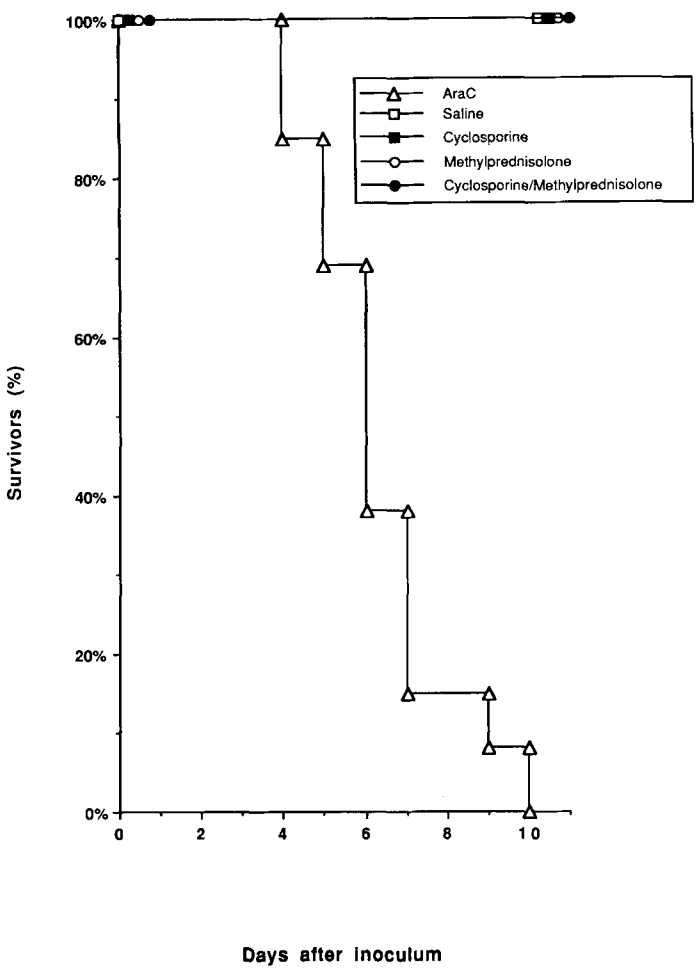

Figure 1. Kaplan-Meier survival curves for normal and immunosuppressed rabbits after a standard intratracheal challenge with 2.8×10^8 CFU of *A. fumigatus*. Median survival was 5 d (range, 3 to 9 d) in granulocytopenic (AraC) rabbits (n = 13). In comparison, no mortality was observed among rabbits treated with saline (n = 12), cyclosporine alone (CsA) (n = 15), methylprednisolone alone (MP) (n = 9), and CsA+MP (n = 12) ($p < 0.01$).

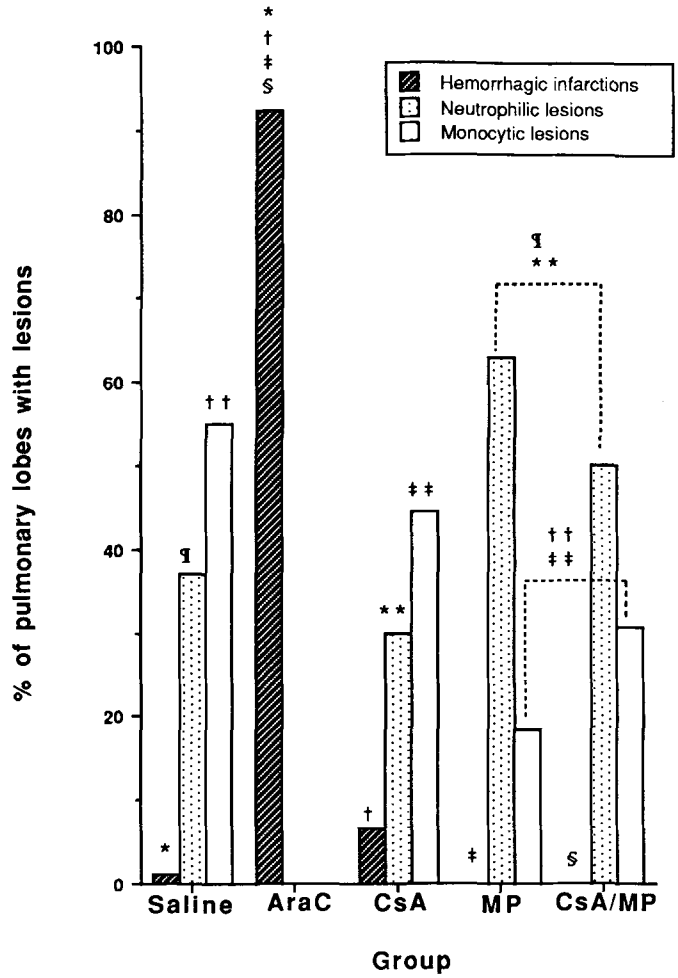

Figure 2. Pulmonary lesions in normal and immunosuppressed rabbits with invasive aspergillosis. Pulmonary lesions in granulocytopenic rabbits (AraC) (n = 13) were predominantly hemorrhagic infarcts (*, †, ‡, § $p < 0.001$), with a conspicuous absence of neutrophilic and monocytic lesions. Rabbits immunosuppressed with methylprednisolone (MP) (n = 9) or cyclosporine plus methylprednisolone (CsA+MP) (n = 12) had significantly more neutrophilic lesions (¶, ** $p < 0.02$) and less monocytic lesions († †, ‡ ‡ $p < 0.01$) than did rabbits treated with cyclosporine alone (CsA) (n = 15) or saline (n = 12).

RESULTS

Patterns of Acute Pulmonary Aspergillosis during Immunosuppression

The patterns of aspergillosis during immunosuppression caused by the different immunosuppressive regimens were measured by survival, type of macroscopic and microscopic pulmonary lesions, lung weight, results of cultures for *A. fumigatus*, and the presence of culture or histologically proved extrapulmonary aspergillosis.

The survival curves for the different groups of rabbits after a standard intratracheal challenge with 2.8×10^8 *A. fumigatus* conidia are shown in Figure 1. The survival curve declined rapidly in granulocytopenic rabbits, with median survival of 5 d (range, 4 to 10 d). In comparison there was no mortality among the rabbits included in other experimental groups ($p < 0.01$).

Pulmonary lesions in granulocytopenic rabbits were predominantly hemorrhagic infarcts ($p < 0.001$), with a conspicuous absence of neutrophilic and monocytic lesions (Figure 2). Rabbits immunosuppressed with MP alone or in combination with CsA

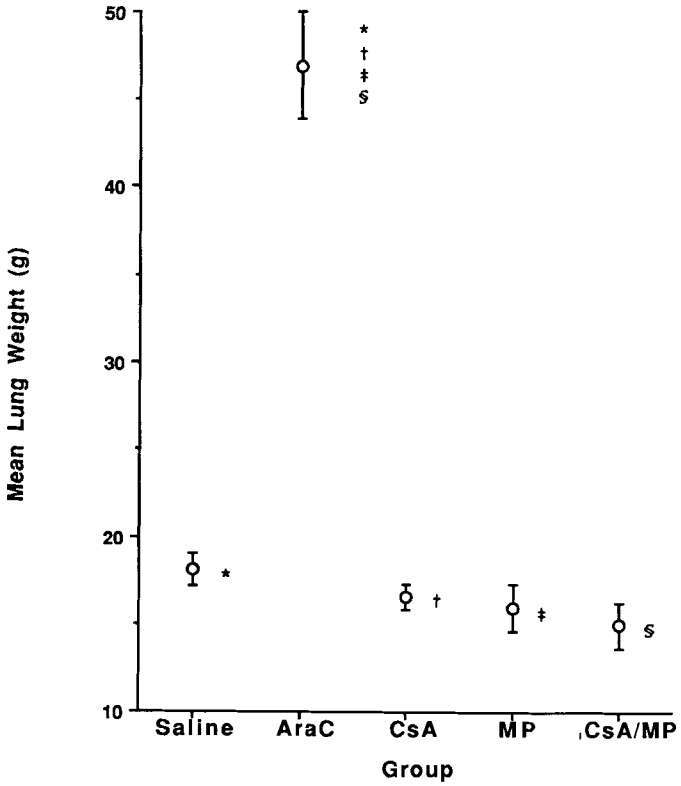

Figure 3. Lung weights (mean ± SEM) in normal and immunosuppressed rabbits with invasive pulmonary aspergillosis. The lungs of granulocytopenic rabbits (AraC) (n = 13) weighed 2.5 to 3 times more than the lungs of the rabbits treated with saline (n = 12), cyclosporine alone (CsA) (n = 15), methylprednisolone alone (MP) (n = 9), and CsA+MP (n = 12) (*, †, ‡, §, $p < 0.01$).

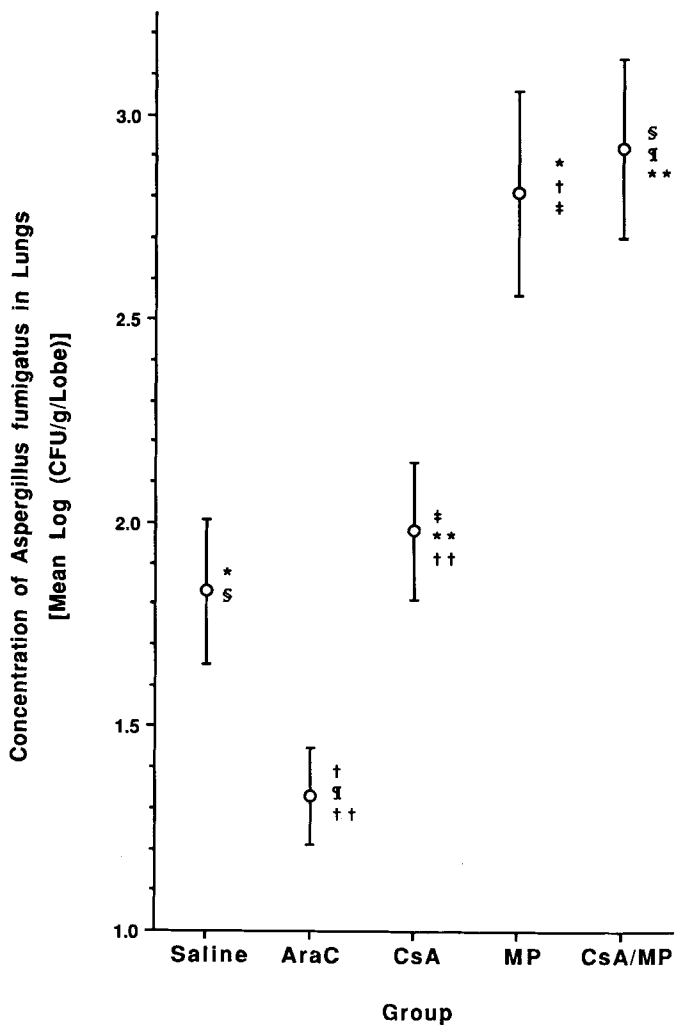

Figure 4. Quantitative lung tissue (mean log CFU/g/lobe ± SEM) cultures in experimental pulmonary aspergillosis. The tissue burden of *A. fumigatus* was greatest in rabbits treated with methylprednisolone with or without cyclosporine (MP and CsA+MP) (n = 21) in comparison with those treated with AraC (n = 13), saline (n = 12), or cyclosporine alone (CsA) (n = 15) (‡, ** $p < 0.05$), (*, †, §, ¶ $p < 0.01$). There was no significant difference between AraC-treated and saline-treated animals.

had significantly more neutrophilic lesions ($p < 0.02$) and fewer monocytic lesions ($p < 0.01$) than did both those treated with CsA and saline-treated control animals. The lungs of granulocytopenic rabbits weighed 2.5 to 3 times more than the lungs of rabbits that were normal or had been treated with CsA with or without MP ($p < 0.01$) (Figure 3). The increase weight of the lungs correlated directly with the number of hemorrhagic infarctions. Hemorrhagic infarcts were not observed in nongranulocytopenic rabbits.

The tissue burden of *A. fumigatus* was greatest in rabbits treated with MP alone or in combination with CsA (Figure 4). The tissue burden of *A. fumigatus* was six to 30 times greater in MP-treated rabbits than in neutropenic, CsA-treated, and saline-treated rabbits ($p < 0.05$). There also was a trend toward higher frequency of positive BAL cultures for *A. fumigatus* in rabbits receiving MP with or without CsA (33%) than in other rabbits (10%) treated with saline, AraC, or CsA ($p = 0.06$). Extrapulmonary aspergillosis was documented postmortem in almost two thirds of granulocytopenic rabbits, a proportion significantly higher ($p < 0.01$) than the one observed in the other groups (Table 1).

Histologic studies revealed two patterns of tissue damage caused by invasive pulmonary aspergillosis (Figures 5 and 6). Hemorrhagic infarction was observed predominantly in granulocytopenic rabbits ($p < 0.001$), and inflammatory necrosis was observed predominantly in nongranulocytopenic immunosuppressed rabbits as well as in saline-treated control rabbits ($p \leq 0.01$) (Table 2). Fully developed hyphae and angioinvasion were

TABLE 1

FREQUENCY OF EXTRAPULMONARY ASPERGILLOSIS

Group	Rabbits Studied (*n*)	Rabbits with Extrapulmonary Aspergillosis (*n*)	(%)
Saline	12	0	0*
AraC	13	8	61.5*,†,¶,§
Cyclosporine	15	0	0†
Methylprednisolone	9	2	22.2¶
Cyclosporine and methylprednisolone	12	0	0§

† $p = 0.001$.
*, § $p = 0.004$.
¶ $p = 0.166$.

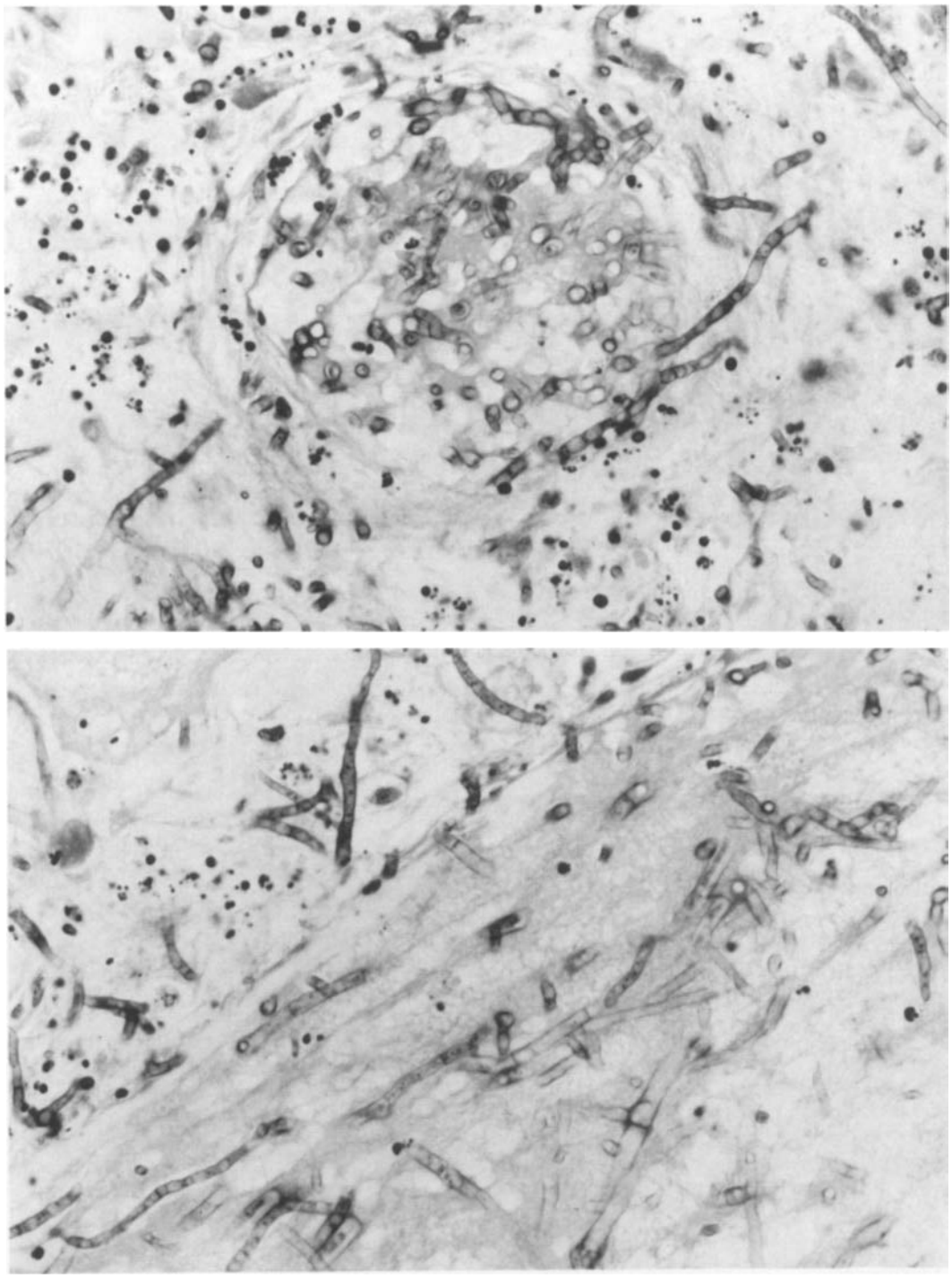

Figure 5. Histopathologic features of invasive pulmonary aspergillosis in granulocytopenic rabbits reveal long, slender dichotomously branching septate hyphae of *Aspergillus* with a paucity of surrounding cellular inflammatory infiltrate (*upper panel:* periodic acid-Schiff stain, original magnification: ×630) and invasion of pulmonary blood vessels by hyphae of *Aspergillus* resulting in intravascular thrombosis (*lower panel:* periodic acid-Schiff stain, original magnification: ×630).

found predominantly in granulocytopenic rabbits ($p \leq 0.004$), whereas conidia in different stages of germination were the predominant fungal structures seen in nongranulocytopenic rabbits.

Patterns of Chronic Invasive Pulmonary Aspergillosis during Immunosuppression

There were no deaths during the 28-day period of observation after the intratracheal inoculation with *A. fumigatus* conidia. Neutrophilic lesions were observed more frequently in rabbits immunosuppressed with MP with or without CsA than in rabbits treated with CsA alone ($p = 0.002$) or with saline ($p = 0.04$). Postmortem studies showed no difference in lung weight between groups. In comparison with rabbits killed 12 d after the inoculum, animals killed 28 d after inoculum continued to clear organisms (Figure 7). There were, however, significantly slower rates of clearance in the animals immunosuppressed by MP and

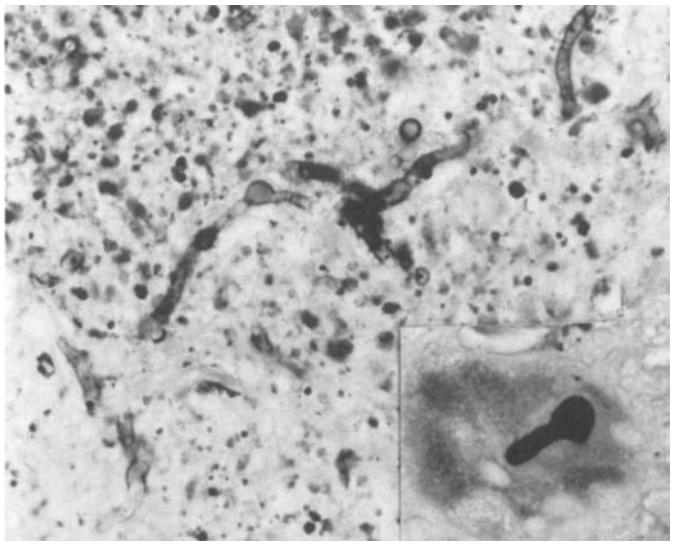

Figure 6. Histopathologic features of invasive pulmonary aspergillosis in rabbits immunosuppressed by methylprednisolone and cyclosporin A (MP+CsA) reveal damaged, truncated, and irregularly shaped hyphae of *Aspergillus* surrounded by tissue necrosis, pyknotic nuclei, and inflammatory cellular debris (periodic acid-Schiff stain, original magnification: ×630). The inset demonstrates germinating *Aspergillus* conidia, which were seen frequently in nongranulocytopenic MP+CsA rabbits, but not in granulocytopenic rabbits (methenamine silver stain, original magnification: ×1,000).

MP + CsA. The tissue burden of *A. fumigatus* was lowest in both normal and CsA-treated rabbits ($p < 0.01$). There was a higher pulmonary concentration of *A. fumigatus* in rabbits treated with MP + CsA than in rabbits treated with MP alone ($p < 0.05$). Less than 10% of pulmonary lobes in rabbits treated with saline (two of 24) or with CsA alone (one of 18) were culture-positive for *A. fumigatus* versus 62.5% (15 of 24) and 83% (20 of 24) in rabbits immunosuppressed with MP with or without CsA, respectively ($p < 0.01$).

DISCUSSION

There were striking differences in patterns of infection and inflammation of invasive pulmonary aspergillosis according to the type of underlying immune defect. Rabbits immunosuppressed by persistent granulocytopenia had high mortality, hemorrhagic infarctions, excessive lung weights, hyphal invasion of large blood vessels, and extrapulmonary dissemination. Virtually all detectable fungal elements in granulocytopenic rabbits were hyphae. By comparison, rabbits immunosuppressed by MP + CsA but challenged with the same respiratory inoculum had low mortality, extensive inflammatory necrosis with neutrophilic and monocytic lesions, virtually no hemorrhagic infarctions, only slightly elevated lung weights, and low frequency of dissemination. There were significantly fewer hyphae and more partially germinated and nongerminated conidia in rabbits treated with MP and/or CsA than in granulocytopenic animals. Methylprednisolone was the major immunosuppressive agent in rabbits treated with MP + CsA. Cyclosporin A in the highest tolerated doses alone did not contribute to progression of invasive aspergillosis. However, CsA added to MP over 28 d further increased the microbiologic tissue burden of *Aspergillus fumigatus*.

In the model of invasive pulmonary aspergillosis in granulocytopenic rabbits, conidia germinated into hyphae, which invaded distal bronchial walls and pulmonary blood vessels, with resultant vascular thrombosis. Angioinvasion and thrombosis by hyphae at discrete foci often resulted in extensive pulmonary infarction and intraalveolar hemorrhage far beyond the focus of vascular invasion. Thrombocytopenia was an important factor contributing to the hemorrhagic component of these pulmonary infarcts in pancytopenic hosts. This pattern of hemorrhagic infarction in granulocytopenic rabbits likely accounts for the higher mortality and greater lung weights at a lower tissue burden in comparison with CsA + MP-treated rabbits. Angioinvasion introduced hyphal elements into major blood vessels and permitted dissemination to extrapulmonary sites, as reflected by more frequent dissemination in granulocytopenic rabbits compared with that in other groups. These experimental findings mirror the evolution of invasive pulmonary aspergillosis in patients with hematologic malignancies and chemotherapy-induced bone marrow aplasia. The hallmark of invasive pulmonary aspergillosis in granulocytopenic patients in tissue and vascular invasion by hyphae, with secondary thrombosis, hemorrhagic infarction, and frequent extrapulmonary involvement (3, 4, 6, 7). Thrombocytopenia in these patients is also a major factor contributing to fatal hemoptysis and pulmonary hemorrhage (26, 27).

Rabbits immunosuppressed with CsA + MP receiving the same intratracheal inoculum of *A. fumigatus* developed an indolent pneumonitis without hemorrhagic infarction. In this model, germinating conidia were abundant, but fully mature hyphae were significantly reduced in comparison with the extensive hyphal infiltration and angioinvasion in granulocytopenic animals. This persistence of germinating conidia and paucity of hyphae is consistent with findings in other nongranulocytopenic and cor-

TABLE 2

EFFECT OF DIFFERENT IMMUNOSUPPRESSIVE REGIMENS OF THE HISTOPATHOLOGIC PATTERNS OF EXPERIMENTAL PULMONARY ASPERGILLOSIS

		Rabbits with					
Group	Rabbits Studied (*n*)	Conidia (*n*) (%)	Germinating Conidia (*n*) (%)	Hyphae (*n*) (%)	Angioinvasion∞ (*n*) (%)	Hemorrhagic Infarction (*n*) (%)	Inflammatory Necrosis (*n*) (%)
Saline	10	10 100*	7 70†	0 0¶	0 0§	0 0**	9 90††
AraC	8	0 0	0 0	8 100	8 100	8 100	0 0
Cyclosporine	15	15 100*	7 47 NS	0 0¶	0 0§	0 0	10 67††
Methylprednisolone	12	12 100*	9 75†	2 17¶	0 0§	0 0**	9 75††
Cyclosporine and Methylprednisolone	12	12 100*	11 92†	3 25¶	0 0§	0 0**	11 92††

*,†,¶,§, **,††, $p \leq 0.01$ for each experimental group in comparison with AraC-treated group.
NS = not significant.

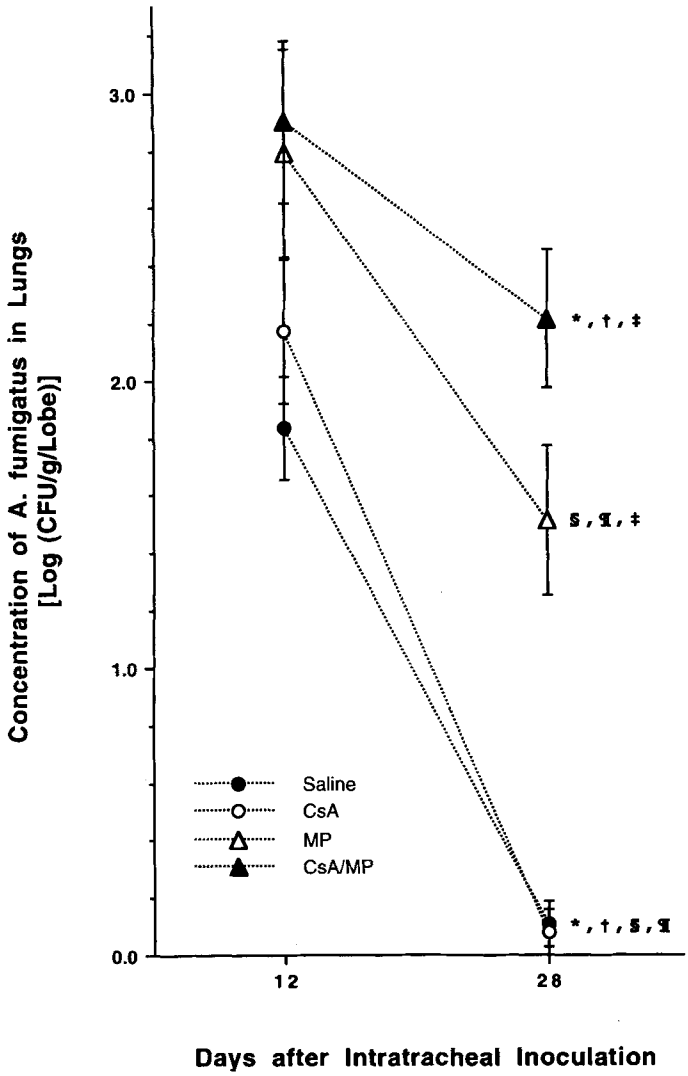

Figure 7. Clearance of *A. fumigatus* from lung tissue in chronic invasive pulmonary aspergillosis. In comparison with rabbits killed at 12 d, rabbits killed at 28 d continued to clear *A. fumigatus* from lung tissue. There were slower rates of clearance in rabbits treated with methylprednisolone (MP) (n = 4) and cyclosporine plus methylprednisolone (CsA+MP) (n = 4) than in rabbits treated with saline (n = 4) or cyclosporine (CsA) (n = 3) (*, †, §, ¶ $p < 0.01$), (‡ $p < 0.05$).

ticosteroid-treated animal models of invasive pulmonary aspergillosis (27–29).

The finding of different mechanisms of tissue injury between profoundly granulocytopenic rabbits and rabbits immunosuppressed with CsA+MP substantiates the concept of two lines of defense against invasive aspergillosis. The first line of defense, which is mediated by pulmonary alveolar macrophages and directed against conidia, can be suppressed by pharmacologic doses of corticosteroids (19–21). Neutrophils are the second line of defense. They are directed against *Aspergillus* hyphae and are abrogated by cytotoxic chemotherapy (21, 30). The critical role of angioinvasion, hemorrhagic infarction and intrapulmonary hemorrhage as mechanisms of tissue injury in neutropenic versus nonneutropenic animals has, however, not been previously illustrated. Moreover, the role of CsA in the immunopathogenesis of invasive pulmonary aspergillosis has not, to our knowledge been previously described.

Rabbits treated in this study with CsA at does of 10 to 20 mg/kg/d and inoculated intratracheally with *A. fumigatus* developed a pattern of necrotizing pneumonitis similar to that of the normal infected control rabbits and significantly less severe than that observed in rabbits treated with MP or CsA+MP. Moreover, rabbits treated with CsA as well as the normal infected control rabbits, as opposed to those receiving MP or CsA+MP, were able to clear the infection. Invasive pulmonary aspergillosis often develops in allogeneic tissue transplant recipients who are receiving concomitant corticosteroid and CsA immunosuppressive therapy for prevention of graft rejection. Little is known about the relative contributions of corticosteroids and CsA to this immunosuppression. Our findings indicate that in this model of invasive pulmonary aspergillosis the corticosteroid component of this regimen is the major immunosuppressive agent.

The dosage of CsA used in these studies (10 to 20 mg/kg/d) clearly compromises cell-mediated immunity in rabbits and is comparable by attainable serum levels to dosages used in human transplant recipients. For example, dosages of 10 to 15 mg/kg/d have been used previously in rabbits for prevention of graft versus host disease after bone marrow transplantation (31), for prophylaxis of rejection after skin allografts (32), and for potentiating development of experimental cryptococcal meningitis (33). Despite these effects in impairment of cell-mediated immunity, our findings revealed that CsA has little effect in predisposing the host to invasive aspergillosis. Cyclosporin A causes immunosuppression by the inhibition of CD4+ T-cell activation and the inhibition of cytokine production that indirectly affects other components of the immune system (34). This mechanism of action, at commonly employed dosages, has little effect on the function of macrophages and granulocytes, the cornerstones of host defense mechanisms against aspergillosis.

Animals treated with MP of CsA+MP had significantly greater tissue burden, higher frequency of lesions, and less effective clearance of *A. fumigatus*. Rabbits treated with CsA alone had patterns of infection similar to that of normal rabbits. Nevertheless, there may be some enhancement of chronic MP immunosuppression by CsA. Animals treated with CsA+MP for 28 d, but not 12 d, had a slightly greater tissue burden of *A. fumigatus* than did rabbits receiving methylprednisolone alone. The finding that MP is the principal drug of the CsA+MP combination, which predisposes to pulmonary aspergillosis, provides a rationale for withdrawal of corticosteroids in order to restore host defense mechanisms while maintaining CsA to reduce the risk of rejection when treating transplant patients with life-threatening *Aspergillus* infections. The management of invasive pulmonary aspergillosis in CsA+MP-immunosuppressed patients, however, is further complicated by potential untoward pharmacologic interactions of CsA with both amphotericin B and antifungal azoles. Amphotericin B increases nephrotoxicity of CsA (35).

Thus, different immunosuppressive regimens cause striking differences in patterns of infection and inflammation of invasive pulmonary aspergillosis. These different patterns may carry important implications for host defense, pathogenesis, immune reconstitution, and antifungal therapy.

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References

1. Pennington, J. E. 1980. *Aspergillus* lung disease. *Med. Clin. North Am.* 64:475–490.
2. Rinaldi, M. G. 1983. Invasive aspergillosis. *Rev. Infect. Dis.* 5:1061–1077.
3. Bodey, G. P. 1966. Fungal infections complicating acute leukemia. *J. Chronic Dis.* 19:667–687.

4. Pannuti, C. S., R. Gingrich, M. A. Pfaller, C. Kao, and R. P. Wenzel. 1992. Nosocomial pneumonia in patients having bone marrow transplant. Attributable mortality and risk factors. *Cancer* 69:2653–2662.
5. Meyer, R. D., L. S. Young, D. Armstrong, and B. Yu. 1973. Aspergillosis complicating neoplastic disease. *Am. J. Med.* 54:6–15.
6. Walsh, T. J. 1990. Invasive pulmonary aspergillosis in patients with neoplastic diseases. *Semin. Respir. Infect.* 5:111–122.
7. Anaissie, E. 1992. Opportunistic mycoses in the immunocompromised host: experience at a cancer center and review. *Clin. Infect. Dis.* 14 (Suppl. 1):S43–S53.
8. Karp, J. E., P. A. Burch, and W. G. Merz. 1988. An approach to intensive antileukemia therapy in patients with previous invasive aspergillosis. *Am. J. Med.* 85:203–206.
9. Meyers, J. D. 1990. Fungal infections in bone marrow transplant patients. *Semin. Oncol.* 17:10–13.
10. Gerson, S. L., G. H. Talbot, S. Hurwitz, and B. I. Strom. 1984. Prolonged granulocytopenia. The major risk factor for invasive pulmonary aspergillosis in patients with acute leukemia. *Ann. Intern. Med.* 100:345–351.
11. Torre-Cisneros, J., R. Mañez, S. Kusne, M. Alessiani, M. Martin, and T. E. Starzl. 1991. The spectrum of aspergillosis in liver transplant patients: comparison of FK 506 and cyclosporine immunosuppression. *Transplant. Proc.* 23:3040–3041.
12. Kusne, S., J. Torre-Cisneros, R. Mañez, W. Irish, M. Martin, J. Fung, R. L. Simmons, and T. E. Starzl. 1992. Factors associated with invasive lung aspergillosis and the significance of positive *Aspergillus* cultures after liver transplantation. *J. Infect. Dis.* 166:1379–1383.
13. Hummel, M., U. Thalmann, G. Jautzke, F. Staib, M. Seibold, and R. Hetzer. 1992. Fungal infections following heart transplantation. *Mycoses* 35:23–24.
14. Chugh, K. S., V. Sakhuja, and S. Jain. 1992. Fungal infections in renal allograft recipients. *Transplant. Proc.* 24:1940–1942.
15. Shah, P. M., and G. Just. 1989. Fungal infections in organ transplantations. *In* K. Holmberg and R. Meyers, editors. Diagnosis and Therapy of Systemic Fungal Infections. Raven Press, New York. 71–79.
16. Rifkind, D., T. L. Marchioro, S. A. Schneck, and R. B. Hill. 1967. Systemic fungal infections complicating renal transplantation and immunosuppressive therapy. *Am. J. Med.* 43:28–38.
17. Bach, M. C., A. Sahyoun, J. L. Adler, R. M. Schlesinger, J. Breman, P. Madras, F. P'eng, and A. P. Monaco. 1973. High incidence of fungus infections in renal transplantation patients treated with antilymphocyte and conventional immunosuppression. *Transplant. Proc.* 5:549–553.
18. Plá, M. P., J. Berenguer, J. A. Arzuaga, R. Bañares, J. R. Polo, and E. Bouza. 1992. Surgical wound infection by *Aspergillus fumigatus* in liver transplant recipients. *Diagn. Microbiol. Infect. Dis.* 15:703–706.
19. Merkow, L. P., S. M. Epstein, H. Sidransky, E. Verney, and M. Pardo. 1971. The pathogenesis of experimental pulmonary aspergillosis: an ultrastructural study of alveolar macrophages after phagocytosis of *A. flavus* spores *in vivo*. *Am. J. Pathol.* 62:57.
20. 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.
21. Schaffner, A., H. Douglas, and A. Braude. 1982. Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to *Aspergillus*. *J. Clin. Invest.* 69:617–631.
22. Walsh, T. J., J. Bacher, and P. A. Pizzo. 1988. Chronic silastic central venous catheterization for induction, maintenance, and support of persistent granulocytopenia in rabbits. *Lab. Anim. Sci.* 38:467–471.
23. Kirkland, T. N., and J. Fierer. 1983. Cyclosporine inhibits *Coccidioides immitis in vitro* and *in vivo*. *Antimicrob. Agents Chemother.* 24: 921–924.
24. Osato, M. S., T. J. Roussel, K. R. Wilhelmus, and D. B. Jones. 1983. *In vitro* and *in vivo* antifungal activity of cyclosporine. *Transplant. Proc.* 15:2927–2930.
25. Dawson-Saunders, B., and R. G. Trapp. 1990. Basic and clinical biostatistics. Appleton & Lange, Norwalk, VA.
26. Panos, R. J., L. F. Barr, T. J. Walsh, and H. J. Silverman. 1988. Factors associated with fatal hemoptysis in cancer patients. *Chest* 94:1008–1013.
27. Graybill, J. R., and S. R. Kaster. 1984. Experimental murine aspergillosis. Comparison of amphotericin B and a new polyene antifungal drug, SCH 28191. *Am. Rev. Respir. Dis.* 129:292–295.
28. Sidransky, H., and L. Friedman. 1959. The effect of cortisone and antibiotic agents on experimental pulmonary aspergillosis. *Am. J. Pathol.* 35:169–183.
29. Kurup, V. P., and N. K. Sheth. 1981. Experimental aspergillosis in rabbits. *Comp. Immunol. Microbiol. Infect. Dis.* 4:161–174.
30. Patterson, T. F., P. Miniter, J. L. Ryan, and V. T. Andriole. 1988. Effect of immunosuppression and amphotericin B on *Aspergillus* antigenemia in an experimental model. *J. Infect. Dis.* 158:415–422.
31. Bigelow, C. L., L. T. Adler, and F. R. Appelbaum. 1987. Allogeneic bone marrow transplantation in irradiated adult rabbits. *Transplantation* 44:351–354.
32. Gratwohl, A., I. Forster, and B. Speck. 1982. Histoincompatible skin and marrow grafts in rabbits on cyclosporin A. *Transplantation* 33: 361–364.
33. Perfect, J. R., and D. T. Durack. 1985. Effects of cyclosporine in experimental cryptococcal meningitis. *Infect. Immun.* 50:22–26.
34. Thomson, A. W., J. Woo, and M. Cooper. 1992. Mode of action of immunosuppressive drugs with particular reference to the molecular basis of macrolide-induced immunosuppression. *In* A. W. Thomson, editor. The Molecular Biology of Immunosuppression. John Wiley & Sons, New York. 153–179.
35. Kennedy, M. S., M. J. Deeg, M. Siegel, J. J. Crowley, R. Storb, and E. D. Thomas. 1983. Acute renal toxicity with combined use of amphotericin B and cyclosporine after marrow transplantation. *Transplantation* 35:211–215.