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

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ABSTRACT By comparing natural immunity to Aspergillus fumigatus (AF) in vivo with the action of human or mouse phagocytes against AF in vitro, we delineated two sequential lines of defense against AF. The first line of defense was formed by macrophages and directed against spores. Macrophages prevented germination and killed spores in vitro and rapidly eradicated conidia in vivo, even in neutropenic and athymic mice. The second was the neutrophilic granulocyte (PMN), which protected against the hyphal form of AF. Human and mouse PMN killed mycelia in vitro. Normal, but not neutropenic mice, stopped hyphal growth, and eradicated mycelia. Either line of defense acting alone protected mice from high challenge doses. Natural immunity collapsed only when both the reticuloendothelial system and PMN were impaired. These findings are in keeping with the clinical observation that high doses of cortisone and neutropenia are the main risk factors for invasive aspergillosis. Cortisone inhibited the conidiodal activity of mouse macrophages in vivo and of human or mouse mononuclear phagocytes in vitro. Cortisone damaged this first line of defense directly and not through the influence of T lymphocytes or other systems modifying macrophage function as shown in athymic mice and in vitro. In addition, daily high doses of cortisone in mice reduced the mobilization of PMN so that the second line of defense was also impaired. Thus, cortisone can break down natural resistance on its own. Myelosuppression rendered mice susceptible only when the first line of defense was overpowered by high challenge doses, by activated spores that cannot be killed by macrophages, or by cortisone suppression of the conidiodal activity of macrophages.

The host, thus, can call upon two independent phagocytic cell lines that form graded defense systems against aspergillus. These lines of defense function in the absence of a specific immune response, which seems superfluous in the control and elimination of this fungus.

INTRODUCTION

Invasive aspergillosis, due mainly to Aspergillus fumigatus (AF), is a serious problem in immunocompromised patients, especially those with hematologic neoplasia, renal transplants, and chronic granulomatous disease (1-5). The respiratory tract is generally regarded as the portal of entry for invasive aspergillosis because the lung is frequently infected (1, 2), the disease is prevented by filtration of the air in the environment of patients at risk (6), and the spores are small enough to reach the alveoli (7). In both the pulmonary and the disseminated disease of brain, kidneys, liver, and other organs, the fungus invades blood vessels and causes thrombosis and infarcts (1, 2).

From clinical observations it has been concluded that neutropenia and high doses of corticosteroids are

1 Abbreviations used in this paper: AF, Aspergillus fumigatus; agar; TSB, trypticase soy broth; HBSS, Hanks' balanced salt solution; FCS, fetal calf serum; PS, penicillin-streptomycin; CA; cortisone acetate; HN₂, nitrogen mustard; HCS, hydrocortisone; CFU, colony-forming units; PMN, polymorphonuclear granulocyte; Δ, difference between two mean values; LD₅₀, median lethal dose.

Received for publication 13 August 1981 and in revised form 4 November 1981.
the two major risk factors for invasive aspergillosis (1, 2) and that the combination may act synergistically to break down natural resistance (2). The high incidence of aspergillosis in chronic granulomatous disease (5, 8) brings out the importance of phagocytes in resistance to aspergilli, but does not discriminate between the relative contributions of polymorphonuclear and mononuclear phagocytes because both are defective in oxidative killing in this disorder (9). Despite this clinical evidence, it has not yet been shown that phagocytes can kill either spores (10) or mycelia of AF in vitro, but there is evidence that polymorphonuclear granulocytes (PMN) can damage hyphae (11).

Cortisone lowers the normally high resistance of laboratory animals to invasive aspergillosis (12–24). For example, mice become extremely susceptible to aspergillosis after large doses of cortisone (13, 14, 23, 24), but not after myelosuppression or neutropenia (14, 23). Cortisone suppresses the accumulation of phagocytes at inflammatory sites, reduces bactericidal and candidicidal activity of monocytes, and impairs expression of cell-mediated immunity (25–27). Although none of these actions of cortisone that might affect aspergillosis have been studied systematically, the interesting observation was made that spores of Aspergillus flavus germinated faster in alveolar macrophages of cortisone-treated mice (22, 28).

In order to clarify the mechanisms of action of cortisone and to identify the role of myelosuppression and neutropenia in lowering resistance to aspergillus infection, we have conducted experiments in a mouse model of invasive aspergillosis. Our findings, which also include observations made during direct comparisons in vitro of human and mouse phagocytes, allow us to delineate two lines of cellular defense that must be overcome by AF in order to establish disseminated infection.

**METHODS**

**Organism**

A strain of Aspergillus fumigatus (AF) isolated from the sputum of a patient at the University Hospital of San Diego was used for all experiments. Conidia were scraped with a wire loop from 4- to 6-d cultures on Sabouraud dextrose agar (SDA) plates supplemented with 100 μg/ml of streptomycin and 100 U/ml penicillin G at 37°C. Conidia were washed three times in 0.9% saline, clumps were disrupted with a Teflon pestle and a Tri-R Stir-R homogenizer (Tri-R Instruments, Inc., Rockville Center, N. Y.), and the suspension filtered through a 15-ml plastic syringe packed with cotton gauze. This procedure gave suspensions of 100% conidia (no mycelia) with > 95% single conidia.

**Preincubated conidia.** In some experiments conidia were preincubated in broth as follows: 5 × 10² filtered single conidia in a volume of 1–5 ml of 0.15 M saline were added to a 500-ml Erlenmeyer flask containing 200 ml of tryptocase soy broth (TSB) supplemented with penicillin-streptomycin (PS) and incubated overnight at room temperature without shaking. After the conidia were collected by centrifugation at 800 g for 15 min, the pellet was washed three times in saline, resuspended, and dispensed into single conidia as described for plate cultures. Phase contrast microscopy showed that preincubated conidia were about four times larger in diameter but still round in form and free of germ tubes.

**Media, drugs, and reagents**

Sabouraud dextrose agar, TSB, and Bacto tryptone were obtained from Difco Laboratories, Detroit, Mich.; thioglycolate from BBL Microbiology Systems, Cockeysville, Md.; cortisone acetate (CA) and nitrogen mustard (HN₂) from Merck Sharp & Dohme, West Point, Pa.; hydrocortisone from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.; Hanks’ balanced salt solution (HBSS), RPMI-1640, Ca²⁺- and Mg²⁺-free phosphate-buffered Dulbecco’s modified Eagle medium, heat-inactivated fetal calf serum (FCS), and lipohylized penicillin-streptomycin (PS) were all purchased from Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y. Amphotericin B was purchased from E. R. Squibb & Sons, Inc., Princeton, N. J., and starch and Isopaque-Ficoll from Sigma Chemical Co., Saint Louis, Mo.

**Animals**

In most experiments we used 6–8-wk-old female CF₁ mice from Charles River Breeding Laboratories, Inc., Wilmington, Mass. 8–10-wk-old nu/nu- and nu/+-BALBc mice were a gift from Dr. N. O. Kaplan from the Athymic Mouse Facility, Cancer Center and the Department of Chemistry, University of California, San Diego. Four to six mice were kept in each cage. Cages and water bottles of immunosuppressed animals were changed three times per week and food and chlorinated drinking water were offered ad lib. Athymic mice were held in a separate room without other animals. Cages, housing material, water, and food were autoclaved before use, and cages were covered with sterile filter caps. The nude mouse wasting syndrome was not observed.

**Immunosuppressive regimens**

**Cortisone.** CA was injected s.c. either in one dose of 5 mg per animal in a volume of 0.2 ml of 0.15 M NaCl solution (saline) 48 h before challenge (15, 24) or in a daily dose of 2.5 mg in 0.1 ml of saline for 6 consecutive d. Myelosuppression was induced by HN₂ dissolved in sterile water according to the directions of the manufacturer and either used within 1 h or frozen at −20°C. Its ability to induce neutropenia was unchanged after 7 d. HN₂ was diluted to a concentration of 200 μg/ml in 0.9% of saline immediately before injection into the lateral tail vein. Optimal myelosuppression was achieved by giving mice 40 μg of HN₂/d on 3 consecutive d before challenge. Only one mouse died from HN₂ without fungal challenge in one of the control groups of five to six mice accompanying each experiment. Larger doses of HN₂ were lethal in the absence of aspergillosis infection.

**Immunized mice**

Mice were immunized with 5 × 10⁶ live conidia, i.v., 28 d before study and were treated with 1 mg/kg of amphotericin B in 5% dextrose, i.v., on days 3, 5, and 7 after the
conidial challenge. This established an infection lasting <28 d. The IgG titer of pooled serum from five mice taken at 28 d, the day of challenge, was >1:1,000, and the IgM titer was >1:100 in an enzyme-linked immunosorbent assay (29) using fragments of boiled mycelia as antigen. Control mice received amphotericin B but no injections of conidia.

**Challenge of mice**

**I.v. model.** Washed conidia were injected in 0.5 ml of 0.9% saline in the lateral tail vein. All challenge doses were standardized by adjusting the concentration of conidia by hematocytometer counts and culturing serial dilutions in TSB.

**Inhalation model.** Mice were placed in the inhalation chamber of Piggot and Emmons (30), which was manufactured by the glass shop of the University of California, San Diego. The chamber permitted simultaneous exposure of 10 animals to conidia aerosolized by blowing air over a culture growing in the chamber on Sabouraud dextrose agar with PS. The challenge dose was determined from the number of colony-forming units (CFU) recovered from the lungs of at least five animals per experiment (see below) and was uniform for each experiment (±0.3 log SD). The exposure time was three min, and the challenge dose was varied by using young cultures (3–4 d, 37°C) for low doses and older cultures (up to 10 d, 37°C) for higher doses.

**Clearance of fungi from tissues**

At specified times animals were killed with ether, and individual organs were homogenized with Teflon pestles and a Tri-R Stir-R homogenizer (Tri-R Instruments, Inc.). Serial 1:10 dilutions of each homogenate were mixed with Sabouraud dextrose agar in pour plates that were read after 48 and 72 h at 37°C. In studies of clearance of inhaled conidia, the right lower lung lobe was not homogenized, but reserved for histology. To calculate the organ distributions, the number of CFU/ml was multiplied by the total volume of homogenate obtained.

**Blood counts, bone marrow smears, and peritoneal exudative response**

Blood obtained from the retroorbital venous plexus (31) with calibrated heparinized glass capillaries was diluted in 0.1 M HCl with 0.1% methylene blue, and leucocytes were counted in a hematocytometer. Differential counts were done on Wright-stained smears, except those below 1000/µl that were made from supravitally stained cells suspended in HCl with methylene blue. Bone marrow aspirated from the femur of killed animals was examined on Giemsa-stained smears. Peritoneal cellular exudate was obtained for cell counts by inserting a pasteur pipette through a small incision, washing with 12 ml of ice-cold Dulbecco's modified Eagle medium free of Ca2+ and Mg2+, centrifuging the collected fluid at 300 g for 10 min at 4°C, and resuspending the cells in 1 ml of Dulbecco's before counting.

**Histology**

Organs were fixed immediately after sacrifice of the animals in 10% phosphate-buffered formalin, pH 7.4, embedded in paraffin and stained by Giemsa's method.

**In vitro studies with phagocytes**

Viability of phagocytes was always >95% as judged by trypan blue exclusion. Cells were counted in 0.1 M HCl with methylene blue in a hemocytometer, and differential counts were made in duplicate from Giemsa-stained smears. Mouse peritoneal macrophages were either resident macrophages obtained without stimulation or exudative macrophages removed 5 d after i.p. injection of 1 ml of 2% starch in saline or 4% thioglycolate in water (31). Resident macrophages were >99% mononuclear cells and induced macrophages >90% mononuclears (<10% PMN). PMN-rich peritoneal exudate cells from mice were removed 4 h after i.p. injection of 1 ml 10% Bacto-Tryptone in saline and contained >68% PMN. Human mononuclear phagocytes were separated by a standard technique (31) from the freshly voided dialysis fluid of four patients with end-stage renal disease without peritonitis; they were not receiving steroids. 2.5 × 109–1 × 1010 cells with 64–87% mononuclear cells were recovered from 2 liter of dialysis fluid. Human PMN were removed by dextran sedimentation from heparinized blood (32) and used directly at a purity of >68% PMN or after purification by the Isopaque-Ficoll technique (32) to a purity of >96%.

**Germination rate of conidia ingested by peritoneal macrophages in vitro**

Peritoneal macrophages were washed three times in HBSS, adjusted to a final concentration of 1 × 106 cell/ml in RPMI-1640 with 10% FCS and 100 U/ml penicillin and 100 µg/ml streptomycin. Aliquots of 0.15 ml were placed on 18-mm round glass coverslips in 60-mm plastic dishes and incubated for 2 h at 37°C. The dishes were washed three times by vigorous rocking with HBSS to remove non-adherent cells and incubated in RPMI with FCS and PS overnight. After three more washes with HBSS, the macrophages were cultured for 48 h longer either with or without 7.5–30 µg/ml of hydrocortisone. These concentrations are below the expected plasma levels of mice given 5 mg of CA, s.c. (35), and comparable to those reached during high-dose steroid treatment of man (34, 35). Thereafter the cells were washed twice more with HBSS and infected with conidia suspended in 5 ml of RPMI with FCS and PS at a concentration of 1–3 × 106/60-mm dish. After 1 h the dishes were vigorously washed six to eight times with HBSS until no extracellular conidia were seen under phase contrast microscopy. At specified times, cell cultures were fixed in 95% ethanol and Giemsa-stained. The phagocytic index varied between experiments from 3.5 to 13 (n macrophages/n conidia) with zero to four conidia per single cell, whether cells were exposed to cortisone or not. Confluence of the macrophage cultures (10–50%) and estimated cell densities were also not influenced by HCS. The germination rate was the percentage of conidia forming mycelia among 100 counted per coverslip from triplicate wells.

**Microassay for killing of spores by mouse peritoneal macrophages**

Washed, thioglycolate-induced peritoneal macrophages were suspended in RPMI-1640 with 10% FCS and PS at a concentration of 1 × 106 cells/ml. Aliquots of 0.1 ml were added to flat-bottomed cluster wells with a capacity of 350 µl (96-well tissue culture clusters, 0.32 cm², Costar Data Packaging, Cambridge, Mass.). After 2 h of incubation, wells
were washed three times with HBSS and incubated overnight with 100 µl of RPMI with FCS and PS, washed again three times with HBSS, and then infected with 100 µl of a suspension of 25–250 CFU single conidia per ml of medium containing FCS. To control for killing by medium alone, conidia were dispensed in each experiment to an additional plate that did not contain macrophages. The medium did not kill the fungus; instead it promoted growth. After 1 h of phagocytosis, the wells containing macrophages were washed seven times with HBSS to remove extracellular spores. Culture medium was added to alternate wells, and 100 µl of distilled water was added to the remainder to lyse the cells and quench the interaction of phagocytes and spores. After 15 min, these control cultures were examined for lysis by phase contrast microscopy and 100 µl of double strength TSB with PS was added to promote fungal growth. In test wells macrophages and conidia were cultured together for 48 h before their interaction was quenched by lysis as described for the control wells with lyzed macrophages. The plates were inspected daily for fungal colonies forming after quenching. Sporulation and spread to other wells was prevented by placing a drop of 5% aqueous methylene blue in all wells showing growth, which uniformly appeared 48 h after quenching.

**Assay for killing of mycelia by PMN**

**Tube assay.** From suspensions of 120–250 CFU/ml of preincubated conidia in RPMI with 10% FCS and PS, aliquots of 100 µl were dispensed to 15 × 150-mm flat bottom glass tubes and incubated at 37°C until mycelial growth was seen by phase contrast microscopy (4–6 h). All tubes were centrifuged at 800 g for 15 min to ensure that mycelium adhered to the bottom of the tubes. 1 ml of a suspension 1–3 × 10^6 PMN in RPMI-1640 with 5% autologous, heat-inactivated serum, or medium and serum without PMN was added. In preliminary experiments it was determined that 3 × 10^6 cells were required to cover the bottom of the tubes from the center to the periphery. To establish contact between the leukocytes and the fungus, all tubes were centrifuged at 150 g for 5 min. In addition to the medium control, the effect of pelleting leukocytes onto mycelia was controlled with PMN that were killed by freezing and thawing (31). After 12-h incubation, 2 ml of distilled water was added to all tubes and the mixture vortexed and incubated for 15 min to lyse the PMN. Thereafter, 2 ml of double strength TSB with PS was added to promote fungal growth. Quenched cultures were observed for 4 d, but macroscopic growth invariably appeared as a clearly visible mycelial network within 48 h.

**Micro-well assay.** A similar assay was performed in flat bottomed 350-µl cluster wells. Preincubated conidia were dispensed in a volume of 100 µl of medium, re incubated until mycelial growth was visible, and centrifuged at 800 g for 15 min. The supernatant was aspirated by vacuum through a 23-gauge needle and replaced with a specified number of phagocytes washed three times in 100 µl of RPMI-1640 with 5% FCS, or with medium containing serum alone. The plates were centrifuged at 150 g for 5 min and incubated for 12 h before quenching with 100 µl of distilled water and addition of double strength TSB. The plates were read for growth for 4 d. Methylene blue was added to wells that turned positive to prevent sporulation.

**Statistical analysis**

The median lethal dose (LD_{50}) for mice challenged with conidia was calculated by the method of Reed and Muench (36). The significance of differences observed in the LD_{50} experiments was computed by comparing the frequency of death with 2 × k contingency tables and by using the Bonferroni-X^2 statistics tables for correction of multiple comparisons (37). Differences of pooled data from several LD_{50} experiments were calculated at each dose level because group sizes were unequal from pooling. The results of the in vitro killing assay were also analyzed by 2 × k contingency tables with correction for multiple comparisons. Differences between mean values were determined by t test.

**RESULTS**

**Effects of immunosuppressive regimens on phagocytes.** HN₂ reduced drastically the total blood leukocyte and PMN counts (Fig. 1), and during the PMN nadir, bone marrow cellularity dropped by >70% with a complete absence of the postmitotic pool of the PMN series. In contrast, the PMN counts were unchanged from 48 h up to 8 d after injection of 5 mg CA, while the mononuclear cell counts dropped by 95% for several days. After daily injections of 2.5 mg of CA, the counts of mononuclear cells were further reduced to <50 cell/µl again without decreased PMN counts.

To compare suppression of the cellular inflammatory response by CA and HN₂, the peritoneal exudative response to heat-killed conidia was studied in normal, CA-, and HN₂-treated mice (Fig. 2). This experiment was repeated with live conidia, and similar results were obtained. When mice were treated in this experiment with CA from 2 d before stimulation with spores until cells were harvested, the total cellular response fell 93% below that of normal mice (P < 0.01). PMN fell 95% (P < 0.001), a suppression similar to that in HN₂-treated mice.

**FIGURE 1** Blood leukocyte changes in mice given HN₂. Response to three daily doses of 40 µg HN₂ i.v. Mean±SD from three animals. (■): total white cell count; (Δ): PMN-count; (◇): PMN-counts from representative individual animals from subsequent experiments.
The number of resident peritoneal macrophages recovered after immunosuppression without stimulation with spores was 4.47±0.8×10⁶ (mean±SD from six animals) in HN₂-treated, and 3.95±0.83×10⁶ in CA-treated mice compared with 5.4±1.6×10⁶ in normal mice, demonstrating that the influx of new phagocytes rather than the pool of resident macrophages is affected by CA and HN₂.

Comparative effect of HN₂ and CA on susceptibility to intravenous conidia. One dose of CA 48 h before challenge reduced the LD₅₀ of intravenous challenge by 1.7 log below normal mice (P < 0.01) in both simultaneous comparisons and four pooled experiments (Table I). After continuous immunosuppression with 2.5 mg of CA for 6 d (beginning 2 d before challenge), the LD₅₀ was 2.2 logs lower than that of animals treated only once with CA.

In contrast, neutropenia induced by HN₂ lowered the LD₅₀ by only 0.52 log (0.1 > P > 0.05, Table I). Because HN₂ and CA suppressed inflammation equally, we sought another explanation for the differences in susceptibility to aspergillosis. First, we examined the possibility that CA suppressed the T lymphocytes by challenging athymic mice with AF spores, but found them equally resistant. Moreover, athymic mice were as susceptible to immunosuppression with CA as CF₁ mice (Table I). To exclude the possibility that cortisone might lower resistance by blocking the early humoral immune response, we tested the susceptibility of mice with antibody titers of >1:100, which developed after active infection. They were as susceptible as their non-immunized littermates (Table I). Thus, CA lowers resistance even in the presence of humoral antibody.

We turned next to mononuclear phagocytes as the cortisone-susceptible defense system. We compared the susceptibility of normal, neutropenic, and CA-treated mice to preincubated spores because studies of Aspergillus flavus infections suggested that spores become resistant to macrophages after preincubation in broth (20). Normal mice were only moderately more susceptible to preincubated spores than freshly collected spores (Table I: ∆ log LD₅₀=0.76, P < 0.01). The findings in CA-treated mice were similar (∆ log LD₅₀=0.85, P < 0.01). Neutropenic mice, however, were much more susceptible to preincubated conidia than to fresh conidia (∆ log LD₅₀=1.55, P < 0.001). More important, myelo-suppressed mice were far more susceptible to preincubated spores than normal mice (∆ log LD₅₀=1.32, P < 0.01) but not to resting spores; in fact the HN₂-treated mice became as susceptible to preincubated conidia as CA-treated mice were to resting spores. This ability of preincubated (activated) spores to kill neutropenic mice that resist resting spores indicated that another line of defense can be breached in the absence of PMN by preincubated spores, but not resting spores.

Distribution and clearance of i.v.-injected spores. Further insight into the role of macrophages was gained by comparing the distribution of spores after intravenous injection into normal, HN₂-treated, CA-

[FIGURE 2 Peritoneal cellular inflammatory response to heat-killed conidia. A. Peritoneal cellular response 24 h after intraperitoneal challenge with 10⁷ heat-killed conidia in 1 ml of 0.15 M NaCl. Mean±SD from six animals per group. All cell counts HN₂ vs. controls and CA vs. controls: P < 0.001. B. Response 72 h after injection of spores. 5 mg CA: animals given 5 mg of cortisone acetate s.c. 48 h before challenge. HN₂: 3×40 μg HN₂ i.v. Mean±SD from four animals per group. Mononuclear cells: HN₂ vs. control P < 0.001, CA vs. control P < 0.05. PMN: HN₂ vs. control P < 0.001, CA vs. control NS.]
In contrast to myelosuppression, CA treatment impaired clearance of the fungus, and the CFU rose in
several organs. A significant and uniform impairment occurred in the liver between 24 and 48 h after challenge. Thereafter, CFU varied from >500/ml in infarcted livers to <10 ml in grossly normal livers (Fig. 3A). The fungus always multiplied in the kidneys of CA-treated mice (Fig. 3B), caused cortical lesions with a scant phagocytic response, and finally destroyed the medulla. In contrast, no lesions or fungal growth were seen in the kidneys of HN2-treated or normal mice. AF multiplied in the brains of five of nine CA-treated animals and in the lungs of one. Elimination of the fungus from the spleen was not impaired by CA, and the fungus did not multiply in this organ.

Athymic mice and heterozygote littermates eliminated the fungus at the same rate from the liver (Fig. 4), brain, spleen, and lung. In addition, the elimination pattern of spores from these organs by nu/nu- and nu/+BALBc mice was similar to that of CF1-mice, in spite of the higher challenge dose to athymic mice (2 x 10⁵ vs. 6 x 10⁴). This higher dose caused kidney infection with gross lesions in several nu/nu- and nu/+ mice. Between days 7 and 17 after challenge, >300 CFU/ml of kidney homogenate were recovered from two of nine nu/nu-mice and three of nine nu/+ mice; the other mice eliminated the fungus from the kidneys by the pattern seen in CF1 mice with a lower challenge dose. Even with this protracted infection, there was no difference between athymic mice and phenotypically normal littermates.

Preincubation of spores in broth resulted in a marked change in organ distribution. 2 h after injection of preincubated conidia, more fungus was recovered from the lungs (25%), kidneys (5.5%), and brain (0.5%), and less from the liver (67%) and spleen (2%) than in experiments with resting spores. The altered distribution is attributed to the increased size of spores and might explain the increased susceptibility of normal, CA-treated and, in part, neutropenic mice. In addition to the altered distribution, the clearance of spores was reduced in myelosuppressed mice and the fungus was not eradicated as it was by normal mice. This difference was independent of the number of organisms reaching individual organs (Fig. 5A and B). Thus, myelosuppressed mice that eliminate resting spores at least as well as control mice could not mount an effective defense against preincubated spores. Clearance was delayed in all organs as early as 24 h after challenge and the fungus multiplied, especially in the kidneys and brain. In contrast, normal mice successfully controlled the infection in the kidneys and brain at the lower challenge dose. At the higher challenge dose (2 x 10⁴ preincubated conidia), 75 times more CFU were recovered per milliliter of brain homogenate than in experiments with 6 x 10⁴ resting spores. The higher challenge caused limited growth of the fungus in the brain of control animals but significantly less than in myelosuppressed mice (Fig. 6B) and killed no normal animals by day 14, whereas myelosuppressed animals died within 6 d after challenge. Thus, normal mice controlled the infection even when challenged with 2 x 10⁴ preincubated conidia.

Susceptibility of immunsuppressed mice to inhaled conidia. To find out if i.v. challenge was representative of natural infection, we exposed mice to conidia in an inhalation chamber. Normal mice were extremely resistant to inhaled conidia (Table II) and HN2-treated mice died only at high challenge doses. One dose of CA also had little effect on the lethality of inhaled fungi but continuous immunosuppression with 2.5 mg of CA for 6 consecutive d killed all animals exposed to spores, even at the lowest challenge dose of 2 x 10⁶ CFU (Table II). In contrast to normals, myelosuppressed mice given one dose of CA were vulnerable to challenge with a moderate dose of inhaled conidia. Thus, a challenge with <1.5 x 10⁶ spores killed neither normal nor HN2-treated mice and only one mouse given a single dose of CA, but all mice treated with one dose of CA plus HN2 died. Mice treated with this combined regimen were as susceptible as those given daily injections of CA alone (Table II).

Clearance of inhaled conidia. Normal CF1 mice eliminated conidia at highly consistent rates in three studies with different batches of mice (Fig. 6A). Inhaled conidia were cleared efficiently by severely neu-
tropenic mice as expected from their high resistance to invasive pulmonary aspergillosis (Fig. 6B). The clearance curves of both normal and neutropenic mice follow first order kinetics with no acquired enhancement of elimination that might suggest a specific immune response during the clearance studies. Abundant spores, but no mycelia, were seen in histologic sections of the right lower lobe up to 96 h after challenge. Because spores are prevented from germination and thus are the only recognizable form of the fungus and because >90% of CFU are cleared from the lungs per day, it follows that spores can be cleared efficiently by a mechanism that is independent of neutrophils. Daily cultures of homogenates of brain, spleen, liver,

**TABLE II**

*Susceptibility of Mice to Inhaled Spores of AF*

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* Each challenge dose of spores represents an independent experiment.
† Mice myelosuppressed with 3 × 40 μg of nitrogen mustard.
§ Mice immunosuppressed with a single dose of cortisone acetate.
¶ Mice immunosuppressed with daily 2.5 mg of cortisone acetate for 6 d.
** Number dead at 21 d over the number challenged.
†† P vs. control < 0.01.
and kidneys after inhalation of spores disclosed no fungus in organs other than the lung. Athymic mice cleared inhaled spores as effectively as phenotypically normal littermates (Fig. 6C).

Unlike athymic and myelosuppressed mice, those treated with CA exhibited impaired clearance of AF (Fig. 6D). By 24 h after inhalation, the clearance rate of conidia in CA-treated mice was significantly below normal. At this phase of clearance, conidia represented the predominant form of the fungus in both CA-treated and control mice. By 48 h, the differences between the clearance curves widened, and histologic sections from CA-treated mice showed progressively more mycelia, invasion into blood vessels, and consequent pulmonary infarction. Many spores appeared to be ingested by mononuclear phagocytes, and transformation of spores into mycelia was observed in phagosomes of CA-treated, but not normal or HN2-treated

**FIGURE 6** Clearance of inhaled conidia by normal, myelosuppressed, cortisone-treated, and athymic mice. Clearance of conidia from the lungs after exposure of mice in four independent experiments to aerosolized spores. Mean±CFU/ml of lung homogenate from three mice per group at each time point. Myelosuppressed mice: 3 × 40 μg of nitrogen mustard i.v. Cortisone-treated mice: 6 × 2.5 mg of cortisone acetate s.c. B. All Δ log CFU:NS. C. All Δ log CFU:NS (two tailed t test). D. Δ log CFU at all time points: P < 0.005.
mice. Cultures disclosed that invasive growth in the lungs was followed by dissemination of the fungus to the kidneys, brain, and liver in most CA-treated mice.

Interaction of human and murine macrophages with resting and preincubated spores of AF. Direct evidence for the importance of mononuclear phagocytes as the first line of defense against AF was obtained from studies on the interaction of macrophages with conidia. When spores were injected i.p., they were readily ingested by macrophages whether mice were immunosuppressed or not. When the peritoneal exudate was harvested 24 h after challenge, the spores were still predominantly in macrophages. In three different experiments, CA-treatment significantly impaired the ability of peritoneal macrophages to prevent transformation of spores into mycelia in vivo (Table III). One dose of CA increased the germination rate at 24 h by two- to threefold and daily treatment by 8- to 10-fold. The faster germination was independent of the cellular composition of the peritoneal exudate that was similar in mice treated with HN2 or continuous CA. Because myelosuppressed mice retained the capacity to prevent germination, these experiments pointed to a functional disturbance in mononuclear phagocytes produced by CA.

Macrophages also slowed transformation of ingested spores into mycelia in vitro, but less efficiently than in vivo. Observations on germination rates were not possible beyond 24 h because progressive growth of escaping mycelia destroyed and overgrew the monolayer of phagocytes. Germination rates were highest between 15 and 21 h of co-culture but did not reach a definite plateau at 24 h, indicating that prevention of germination is not synonymous with killing of spores.

Cortisone inhibited the ability of macrophages to prevent germination of spores in vitro also. Germination rates increased significantly by 2.3- to 3.2-fold after resident or induced peritoneal mouse macrophages were exposed to cortisone in vitro or in vivo (Table IV). Resident macrophages, whether exposed to CA or not, were less able to prevent germination; and co-cultures with resident cells had to be evaluated at 12 h because of fungal overgrowth. Direct stimulation of the fungi by cortisone was excluded because no cortisone was present in the culture medium when cells were infected, and germination was not enhanced when cortisone was added to conidia in medium alone (data not shown).

Human macrophages were comparably inhibited by cortisone (Table IV). They also lost the ability to prevent germination of ingested spores after cortisone treatment. The mycelia sprouting from spores pierced the cells and spread rapidly extracellularly.

Macrophages could not prevent transformation of preincubated spores into hyphae. By 24 h after ingestion, very few nongerminated spores could be made out in macrophages, and the abundant mycelial forms of the fungus could no longer be counted individually so that an exact germination rate could not be determined. We solved this technical problem by showing that spores preincubated in tissue culture medium for 6 h before a 10-h period of phagocytosis transformed significantly faster than resting spores subjected to phagocytosis for 24 h. Mean transformation rates ±SD from two independent experiments were 33±3.8% for preincubated spores and 22±2.7% for resting spores (P = 0.005).

Because multiplication of escaping fungi made it impossible to test killing by demonstrating a reduction of CFU, we tried to determine if macrophages could sterilize small conidial inocula in microcultures. In five consecutive experiments, macrophages sterilized a significant number of wells to which small inocula of spores were added. Sterilization occurred in a dose range of 2 to 12 CFU per well and was dose dependent. No sterilization was detected when a dose of 22 CFU was added per well (Table V). In contrast to the findings with resting spores, preincubated conidia were not killed in parallel microculture experiments with the same cell preparations as predicted by the inability of macrophages to prevent their germination (Table V).

Killing of mycelia by human and murine PMN. In view of our in vivo evidence from the susceptibility and clearance studies that neutrophil granulocytes form the important defense line against AF after ger-

### Table III

<table>
<thead>
<tr>
<th>Groups of mice</th>
<th>Germination %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>3.4±1.1</td>
</tr>
<tr>
<td>Cortisone, single dose, 5 mg</td>
<td>10.3±2.3†</td>
</tr>
<tr>
<td>Cortisone, 4 × 2.5 mg</td>
<td>30.7±4.2‡</td>
</tr>
<tr>
<td>Nitrogen mustard, 3 × 40 μg</td>
<td>2.3±1.2&quot;</td>
</tr>
<tr>
<td>Preincubated conidia</td>
<td>28.2±4.3§</td>
</tr>
<tr>
<td>nu/nu BALB,§</td>
<td>3.0±1.6</td>
</tr>
<tr>
<td>nu/nu BALB, 4 × 2.5 mg cortisone¶</td>
<td>26.2±5.2‡</td>
</tr>
</tbody>
</table>

*% Conidia transforming into mycelia in peritoneal macrophages 24 h after i.p. challenge with 2 × 10⁶ conidia (mean±SD from duplicate combined experiments with three to five animals per group in each experiment).
† vs. control P < 0.01.
‡ vs. control P < 0.001.
" vs. control: NS.
¶ Single experiment.
### TABLE IV

*Effect of Cortisone on the Germination Rate of Conidia Ingested by Peritoneal Macrophages In Vitro*

<table>
<thead>
<tr>
<th>Macrophage source</th>
<th>Cortisone exposure</th>
<th>Germination rate exposed cells</th>
<th>Germination rate control cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse, induced†</td>
<td>in vivo‡</td>
<td>50.3±6%§</td>
<td>21.3±2.9%</td>
</tr>
<tr>
<td>Mouse, resident</td>
<td>in vivo‡</td>
<td>41.0±7.3%§</td>
<td>13.7±3.3%†</td>
</tr>
</tbody>
</table>

In vitro

µg HCS/ml

<table>
<thead>
<tr>
<th>Mouse, induced†</th>
<th>30 µg/ml</th>
<th>60.5±5.8%§</th>
<th>20.8±2.1%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.5 µg/ml</td>
<td>30.3±3.2%§</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 µg/ml</td>
<td>37.7±3.5%§</td>
<td>4.7±2.1%†</td>
</tr>
<tr>
<td></td>
<td>30 µg/ml</td>
<td>41.7±5.5%§</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>30 µg/ml</td>
<td>39±3.6%§</td>
<td>10.3±1.7%</td>
</tr>
<tr>
<td>Human</td>
<td>7.5 µg/ml</td>
<td>50.8±3.4%§</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 µg/ml</td>
<td>60.5±5%§</td>
<td>11.8±2.8%</td>
</tr>
<tr>
<td></td>
<td>30 µg/ml</td>
<td>59.6±2.5%§</td>
<td></td>
</tr>
</tbody>
</table>

* Mean±SD of germination rates at 24 h, pooled data from two independent experiments.
† Macrophages harvested 5 d after stimulation with thioglycolate.
‡ 4 Daily injections of 2.5 mg cortisone acetate before cell harvest.
§ vs. control: *P* < 0.001; germination rates of media controls were >90% for each experiment.
¶ Germination rates at 12 h.
** Single experiment.

### TABLE V

*Killing of AF Spores by Mouse Peritoneal Macrophages: Sterilization of Monolayers 48 h after Ingestion of Resting or Preincubated Spores*

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Inoculum size (CFU)</th>
<th>Number of wells sterilized by:</th>
<th>Live macrophages</th>
<th>Quenched (lysed) macrophages</th>
<th>Media controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resting spores</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.6±1.1</td>
<td>47/68*</td>
<td>27/68*</td>
<td>14/56</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2±1.2</td>
<td>26/66*</td>
<td>9/66*</td>
<td>1/26</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.3±2.5</td>
<td>13/48*</td>
<td>1/48*</td>
<td>0/48</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5.7±1.5</td>
<td>10/48†</td>
<td>0/48†</td>
<td>0/48</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>11.7±1.5</td>
<td>5/48§</td>
<td>0/48§</td>
<td>0/48</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>22.3±4.5</td>
<td>0/48</td>
<td>0/48</td>
<td>0/48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Preincubated spores</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5±2</td>
<td>1/48‖</td>
<td>0/48‖</td>
<td>0/48</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7.6±2.5</td>
<td>0/24</td>
<td>0/24</td>
<td>0/48</td>
<td></td>
</tr>
</tbody>
</table>

* *P* < 0.001.
† *P* < 0.01.
‡ *P* < 0.05.
NS for comparisons between corresponding pairs of wells with live vs. quenched (lysed) macrophages, which served as controls for the test wells.

Natural Resistance to Aspergillus 627
mination, we examined human and murine PMN for their ability to kill mycelia in vitro.

In 12 h human blood leukocytes gently centrifuged on top of mycelial micro-cultures in flat bottom tubes sterilized the vast majority of cultures inoculated with up to 26 CFU of conidia. There was no difference in killing between a crude leukocyte preparation (72% PMN; cells vs. media control \( P < 0.001 \)) and purified PMN (96% PMN, cells vs. media control \( P < 0.001 \)). Sterilization did not occur when centrifugation was omitted, the assay was performed in round-bottom tubes, or the leukocytes were killed by freezing or thawing. In a similar assay performed in micro-wells, we proved that it was PMN that killed hyphae by demonstrating that (a) PMN preparations of >99% purity sterilize microcultures, (b) the killing capacity of cell preparations with low PMN fractions is reduced, and (c) mononuclear cells (monocytes and lymphocytes) in excess of the number present in the PMN preparations cannot kill hyphae (Table VI). We obtained similar results with mouse peritoneal leukocytes enriched either for PMN or mononuclear cells (Table VI).

**DISCUSSION**

On the basis of these findings we delineate for the first time two distinct lines of defense against *Aspergillus* and show that both must be breached before the fungus can establish progressive infection. The following discussion recapitulates and synthesizes our findings in support of this premise and relates these experimental results to human aspergillosis.

**Natural resistance directed against spores.** These studies show that natural immunity to resting spores of AF is largely independent of PMN, T lymphocytes, and humoral immunity, and that mononuclear phagocytes form a very efficient defense system by rapidly killing the fungus in its conidial stage (Figs. 3, 4, 6). In keeping with the reports that inhaled spores of *Aspergillus fumigatus* are seen predominantly in phagosomes of alveolar macrophages (22, 28) and that spores of AF are selectively phagocytosed by mononuclear phagocytes in vitro (10), we found that spores of AF are readily ingested by macrophages in vitro and in vivo and preferentially removed by organs rich in reticuloendothelial phagocytes after i.v. injection. We demonstrated both in the peritoneal cavity and in vitro that macrophages of normal mice prevented germination of spores of AF, the prerequisite inhibitory step for killing the fungus in its conidial stage. Finally, we showed that peritoneal macrophages killed small inocula of resting spores in microcultures (Table V).

**Cortisone inhibits killing of spores by macrophages.** Cortisone significantly impaired the ability of macrophages to prevent germination of conidia in vitro (Table IV) and in vivo (Table III), and therefore to kill the spores. Once transformed into a mycelium, the fungus readily pierces the phagocyte in vitro and in vivo. Thus, failure to prevent germination results in impaired clearance of the fungus in vivo (Figs. 3 and 6).

The observation that exposure to cortisone interferes with the capacity of pure cultures of human and mouse macrophages in vitro and of mouse peritoneal macrophages in vivo to prevent germination indicates that the drug acts directly on the mononuclear phagocyte and not through T lymphocytes or other systems that modify macrophage function. Near maximum impairment of human macrophages by 7.5 \( \mu \)g of hydrocortisone per milliliter, a concentration similar to plasma levels achieved by high-dose steroid therapy in humans (34, 35) suggests that conidialcidal activity of human macrophages is also impaired in vivo. This

**TABLE VI**

*Killing of Mycelia by Leukocytes: Sterilization of Mycelial Microcultures after 12 h of Incubation with PMN-rich Preparations*

<table>
<thead>
<tr>
<th>Cell preparations</th>
<th>Inoculum</th>
<th>Media control</th>
<th>Fraction sterilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td>( 3 \times 10^6, &gt;99% )</td>
<td>5±2</td>
<td>3/32</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>( 1 \times 10^6, &gt;99% )</td>
<td>51±8</td>
<td>0/32</td>
</tr>
<tr>
<td>Exp. 3</td>
<td>( 2 \times 10^6, 96% )</td>
<td>11±2</td>
<td>0/48</td>
</tr>
<tr>
<td></td>
<td>( 2 \times 10^5, 72% )</td>
<td>11±2</td>
<td>0/48</td>
</tr>
<tr>
<td></td>
<td>( 2 \times 10^5, 4% )</td>
<td>11±2</td>
<td>0/48</td>
</tr>
<tr>
<td></td>
<td>( 2 \times 10^6, 4% )</td>
<td>11±2</td>
<td>0/48</td>
</tr>
</tbody>
</table>

Mouse peritoneal cell preparations:

| Exp. 4 | \( 3 \times 10^5, 68\% \) | 8±3 | 0/48 | 33/48\( \delta \) |
| Exp. 5 | \( 1 \times 10^5, 78\% \) | 5±3 | 3/48 | 20/48\( \delta \) |
| Exp. 6 | \( 1 \times 10^6, 74\% \) | 3±2 | 9/48 | 44/48\( \delta \) |
| | \( 1 \times 10^6, 7\% \) | 3±2 | 10/44 | 13/44**\( \delta \) |
| Exp. 7 | \( 2 \times 10^5, 73\% \) | 47±12 | 0/48 | 21/48\( \delta \) |
| | \( 2 \times 10^5, 8\% \) | 47±12 | 0/48 | 0/48 |

*Human leukocytes were used either as crude preparations from dextran sedimented blood or after fractionation by the isoaquefificoll technique using a single donor per experiment.

1 PMN-rich exudates were obtained from the peritoneal cavity of mice 4 h after injection of 10% Bacto tryptone and compared to preparations rich in mononuclear cells harvested 5 d after injection of 4% thioglycolate (Exp. 6) or 24 h after injection of 10% Bacto tryptone (Exp. 7).

\( \delta \) vs. control \( P < 0.001 \).

\( \epsilon \) vs. control \( P < 0.001 \).

\( \delta \) vs. control \( P < 0.001 \).

\( * \) vs. control: NS.
is supported by reports that the candidicidal activity of human monocytes is similarly impaired in vitro by exposure to 16 μg/ml of HCS and in vivo by treatment with two doses of 50 mg prednisone per d (26, 27).

Our findings extend the observations of Merkow et al. (22, 28) that macrophages prevented germination of ingested conidia of *Aspergillus flavus* and that conidia enhances intracellular germination, not only by duplicating this phenomenon with AF, but also by discovering three new properties of macrophages: (a) they kill ingested spores (Table V), (b) they protect mice even after ablation of PMN (Tables I and II), and (c) their function is impaired by a direct action of cortisone (Table IV).

**Natural resistance against mycelia.** Although myelosuppression is believed to be the major risk factor for invasive aspergillosis in patients (1, 2), it has only a minor deleterious effect on the natural resistance of laboratory animals and only with extremely high challenges of conidia (14, 23). The potent defense against conidia provided by macrophages in our studies explains these discrepancies. The short term myelosuppression achievable in laboratory animals does not involve the pool of tissue macrophages that has a turnover rate of several months (38) and thus does not affect the function of the reticuloendothelial system.

We have shown that the roles of myelosuppression and neutropenia become evident only after conidia escape from the reticuloendothelial system and start mycelial growth. Overwhelming the macrophages by extremely high doses is not a realistic model of human disease, and it is not surprising that we could not demonstrate a significant difference in the susceptibility of neutrophilic and normal mice when we used this tactic in the i.v. model (Table I). However, as suggested for *Aspergillus flavus* (20), conidia of AF could be rendered resistant to macrophages by preincubation in broth. This simple maneuver revealed an impressive difference in the susceptibility of neutropenic and control mice to aspergillosis and unmasked the importance of the neutrophil granulocyte (Table I).

When we tried to induce mycelial growth in normal animals by challenging them with a high dose of preincubated conidia, mycelia were scarce and surrounded by typical PMN-rich abscesses. In contrast, abundant mycelia invaded the tissues and encountered little or no PMN response in myelosuppressed or CA-treated animals. Cultures of the tissues showed that growth was limited and that fungi were eliminated by normal mice, whereas multiplication was unopposed in immunosuppressed animals (Fig. 5). These findings, along with efficient killing of mycelia by PMN in vitro (Table VI), establish granulocytes as the major defense against mycelia.

**Relative importance of resistance against spores provided by macrophages and against mycelia by PMN.** The strong resistance remaining after ablation of PMN by cytotoxic drugs demonstrates that macrophages can protect animals from high challenge doses of resting spores. When this first line of defense is overpowered with preincubated spores, normal mice can still resist infection, but neutropenic mice are overwhelmed. Thus, neutrophils form a second line of defense by killing mycelia. Natural resistance collapses only when both lines of defense are damaged, as seen after large doses of CA (Tables I and II), which impair killing of conidia by macrophages and the mobilization of PMN around the fungus, or after combined immunosuppression with one dose of CA plus myelosuppression with HN2 (Table II). Thus patients receiving organ transplants, who are at high risk of aspergillosis, receive both high doses of steroids and cytotoxic drugs and are often neutropenic (39). Patients with chronic granulomatous disease are defective in oxidative killing so that their neutrophils would not be expected to kill mycelia nor their mononuclear cells to kill conidia. Old reviews of aspergillosis (1, 2) stem from a time when steroids were part of standard therapy of acute myelogenous leukemia. Lately, steroids are omitted but these patients continue to have serious problems with invasive aspergillosis. This clinical observation does not necessarily contradict our findings that mice immunosuppressed with myelotoxic drugs alone are quite resistant to a challenge with spores, because aplasia induced by treatment of leukemia lasts much longer than that induced in our mice. Aspergillosis is generally seen late in therapeutic aplasia and is not a complication of neutropenic episodes of short duration, an observation in agreement with our experimental findings. Furthermore, bacterial pneumonia often precedes invasive pulmonary aspergillosis (2), another factor that might affect resistance provided by resident pulmonary macrophages.

The disease induced in mice after immunosuppression with a combined regimen of CA and HN2 or high doses of CA alone, which is characterized by invasive fungal growth in the lungs, penetration of pulmonary blood vessels, formation of infarcts and dissemination to the kidneys, brain, and liver, correspond well to the description of human disease (1, 2).

These observations on the existence of alternative systems of cellular defense that can reinforce or replace the other has not been brought into focus before. Joint systems of humoral and cellular defense have been described in resistance to gram-negative bacteria and viruses (40). Against fungi, however, humoral immunity seems to offer little if any resistance (41), so that phagocytic cells are the main barrier against these organisms. It is intriguing that in the absence of effective antibody, the immune system can still call upon
two lines of defense, and that these are two different kinds of phagocytic cells. It is also remarkable that each prong of this double system of cellular resistance is directed at only one of the two stages of the fungus: the macrophage against the spore and the granulocyte against the mycelium.

In contrast to aspergilli, more invasive fungi invade the host even in the presence of intact phagocytic defense systems. These fungi differ from aspergillus by their dimorphism that dispenses with mycelial growth in vivo. This conversion of the mycelial phase to the resistant tissue phase has been observed when spores or mycelia of Coccioides immitis come under attack by macrophages (42) and PMN (43). Control of infection with dimorphic fungi appears to require a specific cell-mediated immune response that is superfluous for monomorphic aspergillus, which are limited to mycelial growth in vivo.

ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Service International Research Fellowship I F05 TW 02935, by the Schweizerische Gesellschaft fuer Innere Medizin, and U. S. Public Health Service training grant AI 07036. We thank the Athymic Mouse Facility, Cancer Center and the Department of Chemistry, University of California, San Diego (supported by grant number CA-23052) for the athymic mice.

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