INFECTION AND IMMUNITY, Dec. 1997, p. 5110–5117 Vol. 65, No. 12
0019-9567/97/$04.00+0

Isolation and Characterization of a Pigmentless-Conidium Mutant of *Aspergillus fumigatus* with Altered Conidial Surface and Reduced Virulence

BERNHARD JAHN,[1]* ANDREAS KOCH,[2] AXEL SCHMIDT,[3] GERHARD WANNER,[4] HEIKE GEHRINGER,[2] SUCHARIT BHAKDI,[1] AND AXEL A. BRAKHAGE[2]

Institut für Medizinische Mikrobiologie der Johannes Gutenberg-Universität, D-55101 Mainz,[1] Lehrstuhl für Mikrobiologie, Universität München, D-80638 Munich,[2] Institut für Chemotherapie, Bayer AG, D-42096 Wuppertal,[3] and Botanisches Institut, Universität München, D-80338 Munich,[4] Federal Republic of Germany

Received 27 May 1997/Returned for modification 29 July 1997/Accepted 14 September 1997

***Aspergillus fumigatus* is an important pathogen of immunocompromised hosts, causing pneumonia and invasive disseminated disease with high mortality. The factors contributing to the predominance of *A. fumigatus* as an opportunistic pathogen are largely unknown. Since the survival of conidia in the host is a prerequisite for establishing disease, we have been attempting to identify factors which are associated with conidia and, simultaneously, important for infection. Therefore, an *A. fumigatus* mutant strain (white [W]) lacking conidial pigmentation was isolated. Scanning electron microscopy revealed that conidia of the W mutant also differed in their surface morphology from those of the wild type (WT). Mutant (W) and WT conidia were compared with respect to their capacities to stimulate an oxidative response in human phagocytes, their intracellular survival in human monocytes, and virulence in a murine animal model. Luminol-dependent chemiluminescence was 10-fold higher when human neutrophils or monocytes were challenged with W conidia compared with WT conidia. Furthermore, mutant conidia were more susceptible to killing by oxidants in vitro and were more efficiently damaged by human monocytes in vitro than WT conidia. In a murine animal model, the W mutant strain showed reduced virulence compared with the WT. A reversion analysis of the W mutant demonstrated that all phenotypes associated with the W mutant, i.e., altered conidial surface, amount of reactive oxygen species release, susceptibility to hydrogen peroxide, and reduced virulence in an murine animal model, coreverted in revertants which had regained the ability to produce green spores. This finding strongly suggests that the *A. fumigatus* mutant described here carries a single mutation which caused all of the observed phenotypes. Our results suggest that the conidium pigment or a structural feature related to it contributes to fungal resistance against host defense mechanisms in *A. fumigatus* infections.**

Aspergillus fumigatus is becoming increasingly important as a causative agent of life-threatening infections in immunocompromised hosts (reviewed in references 7, 10, 13, 34, and 43). During the last 3 decades, the number of invasive *Aspergillus* infections has risen dramatically, and the incidence has been reported to vary between 5 and 19% in transplantation patients (19, 29). *A. fumigatus* is the most prominent pathogen in the *Aspergillus* family, accounting for more than 90% of human *Aspergillus* infections.

Several groups have initiated attempts to identify factors associated with pathogenicity of *A. fumigatus*. Specific interactions of the fungus with fibrinogen and basement membrane laminin, as well as the elaboration of proteases, are being considered in this context (9, 18, 30–32, 37, 38, 42, 44). In addition, production of a cytotoxin by *A. fumigatus* has been studied at the molecular level (1, 2, 23, 33). However, the toxin apparently did not contribute to virulence, as demonstrated in experiments using toxin-deficient mutants (13, 35).

In studies with *Cryptococcus neoformans*, a yeast pathogen which causes life-threatening infections particularly in AIDS patients (51), it has been shown that production of a melanin-like pigment is a virulence-determining factor (20, 21, 46). One mechanism by which pigment might contribute to virulence derives from its ability to confer some resistance to reactive oxygen species (ROS), a major host antimicrobial effector system also active against *Aspergillus* conidia (4, 47, 48). A similar biological function of a fungal pigment has been established for the dematiacous fungus *Wangiella dermatitidis*, a causative agent of phaeohyphomycosis (14, 15).

In aspergillosis, the initial event is the uptake of conidia by the respiratory system. As the survival of conidia and onset of germination are prerequisites for establishing disease, conidial pigmentation could also represent a factor contributing to resistance against host defense mechanisms.

In this study, an *A. fumigatus* mutant strain with loss of conidial pigmentation (white [W]) was generated by UV mutagenesis. Besides a structural change of the conidial wall, the mutant conidia displayed a higher susceptibility to oxidative attack in vitro. This was paralleled by markedly reduced virulence. A reversion analysis of the W mutant strain showed that all phenotypes of W conidia had coreverted in the revertants, strongly suggesting that a single mutation caused all phenotypes in the W mutant strain.

MATERIALS AND METHODS

***Aspergillus* media and growth conditions.** As a minimal medium, the previously described *Aspergillus* minimal medium (AMM) with 1% (wt/vol) glucose as the carbon source was used (8, 26). To obtain distinct colonies, deoxycholate (0.06%, wt/vol) was added to agar plates. *Aspergillus* complete medium agar plates were prepared as previously described (41). *A. fumigatus* strains were grown at 37°C.

Preparation of conidium suspensions. After subculture on Sabouraud agar (Becton Dickinson, Heidelberg, Federal Republic of Germany [FRG]), conid-

* Corresponding author. Mailing address: Institute of Medical Microbiology, Hochhaus am Augustusplatz, D-55101 Mainz, Federal Republic of Germany. Phone: 49-6131-17 28 65. Fax: 49-6131-39 23 59. E-mail: bjahn@mzdmza.zdv.uni-mainz.de.

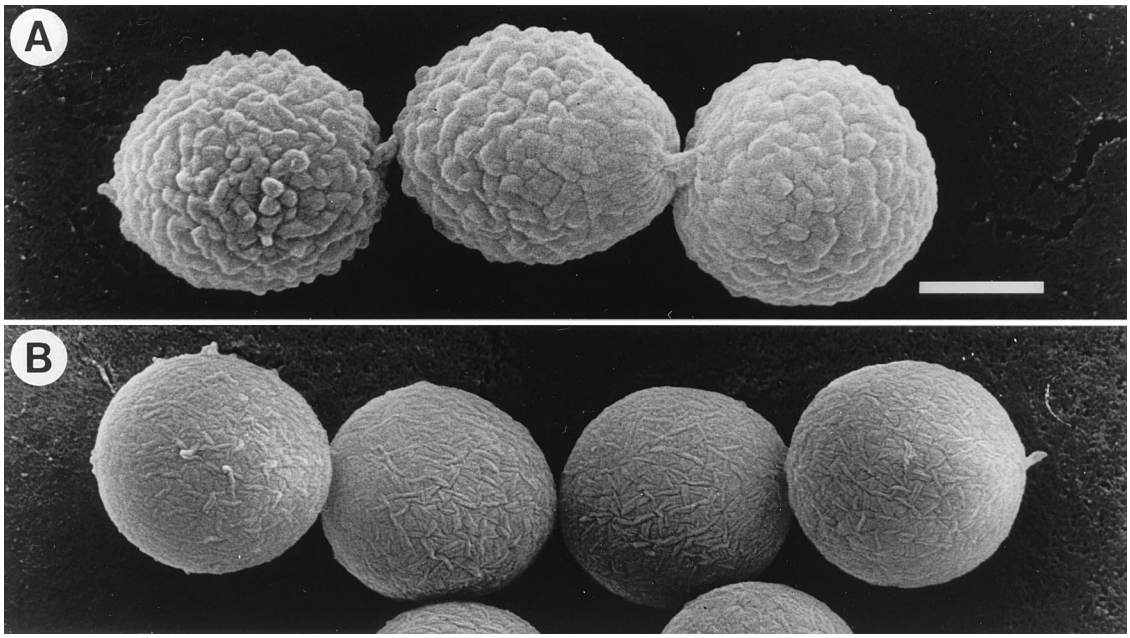

FIG. 1. Scanning electron microscopy photographs of conidia of the WT (A) and W mutant (B) strains. Bar, 1.5 μm.

ium suspensions were prepared as described by Roilides et al. (40). In brief, plates were washed with a physiological saline solution (0.9% [wt/vol] NaCl), and the conidium suspension was filtered twice through a sterile 40-μm nylon mesh (Falcon, Heidelberg, FRG). Penicillin and streptomycin (100 U/ml each) (Antibiotic Mix; Gibco, Karlsruhe, FRG) were added, and suspensions were stored at 4°C.

Mutagenesis. Conidia of the *A. fumigatus* wild-type (WT) strain ATCC 46645 were exposed to 254-nm UV irradiation in 0.1 M $MgSO_4$ solution until the viable count was decreased to 2 to 5%. The irradiated conidium suspension was diluted, plated on AMM agar plates, and incubated at 37°C in the dark. Revertants producing grey-green spores were generated by subjecting W mutant conidia to UV mutagenesis as described above.

Molecular typing. For small-scale preparation of *A. fumigatus* chromosomal DNA, the technique of Raeder and Broder, developed for *Aspergillus nidulans*, was used (36). Molecular typing of *A. fumigatus* strains and mutants was performed essentially as described by Aufauvre-Brown et al. (3). As a primer, R151 (5′-GCTGTAGTGT-3′) was used. PCRs were performed essentially as described by Aufauvre-Brown et al. (3), with the following modifications: the annealing temperature for chromosomal DNA and primers was 30°C, the amount of oligonucleotides used in a 50-μl reaction mixture was 20 pmol, and 2 U of *Taq* polymerase from Eurogentec (Silverstar, Belgium) was used. PCR products were subjected to agarose gel electrophoresis and ethidium bromide staining according to standard procedures. The oligonucleotide R151 gave reproducible products which allowed the *A. fumigatus* strains analyzed to be distinguished.

Scanning electron microscopy. Conidia were plated on slides coated with AMM agar. The slides were incubated at 37°C for 72 h. Colonies were fixed with 2.5% (vol/vol) glutaraldehyde in 75 mM cacodylate buffer (pH 7.0), postfixed with osmium tetroxide, dehydrated in a graded series of acetone solutions, critical-point dried from liquid CO_2, mounted on stubs, and coated with 3- to 5-nm gold-palladium with a magnetron sputter coater. All chemicals were obtained from Sigma (Munich, FRG). The specimens were examined with a Hitachi S-4100 field emission scanning electron microscope operated at 15 kV.

In vitro determination of fungal susceptibility to oxidants. Hydrogen peroxide and sodium hypochlorite were applied as oxidants in phosphate-buffered saline (PBS). Conidium suspensions were adjusted to 10^6/ml in PBS buffer. One hundred microliters of an oxidant and 100 μl of conidium suspension were pipetted in round-bottom microtiter plates (Greiner, Nürtingen, FRG), centrifuged at 1,000 × *g* for 5 min, and incubated at 30°C for 14 h. Thereafter, the fluid was carefully aspirated and replaced by 50 μl of RPMI 1640 (Biochrom, Berlin, FRG) containing 0.5 mg of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (Serva, Heidelberg, FRG) per ml and 100 mM menadione (Sigma). The incubation was continued for another 3 h to allow the formation of formazan by viable conidia (16). Formazan crystals were dissolved by addition of 150 μl of acidic isopropanol (95 ml of isopropanol and 5 ml of 1 N HCl) per well and vigorous shaking for 10 min. Aliquots (150 μl) were transferred to enzyme-linked immunosorbent assay reader plates (Greiner), and measurements were made immediately in a microplate reader (EAR400; SLT, Crailsheim, FRG) at 550 nm. Wells containing the respective oxidant concentrations without conidia served as background controls. Conidia cultivated without oxidants were used as growth controls. The oxidant-induced growth inhibition (GI_{ox}) was determined by the following equation: $GI_{ox} = 1 - [(OD_{ox+s} - OD_{ox})/OD_s]$, where OD_{ox+s} is the optical density in wells after incubation of conidia with oxidant, OD_{ox} is the optical density in wells containing medium and the respective oxidant concentration only, and OD_s is the optical density in wells containing conidia without oxidant as a growth control. Susceptibility to the oxidant was expressed as the 50% inhibitory concentration (IC_{50}), which is the concentration leading to a growth inhibition of 50%.

Preparation of phagocytes. Human polymorphonuclear leukocytes (PMN) were prepared from freshly drawn heparinized blood, and monocytes were isolated from buffy coat as previously described (27, 28).

Measurement of ROS release by phagocytes. Release of ROS was assessed by employing a modified luminol-dependent chemiluminescence assay, as described by Watson et al. (49). Freshly isolated cells were resuspended to give a final concentration of 2.5×10^6/ml in Hanks balanced salt solution–20 mM HEPES buffer (pH 7.3) containing luminol (Sigma) at 125 μM. Two hundred microliters of cell suspension was mixed with 20 μl of conidium suspension in white flat-bottom microtiter plates (Greiner) at effector/target (E/T) ratios of 1:0.16 to 1:10. The plates were placed in a microplate luminometer (Microlumat LB96p; EGG Berthold, Bad Wildbad, FRG) equipped with a temperature device to keep plates at 37°C. Cells treated with 10 nM phorbol-12-myristate-13-acetate (PMA) (Sigma) served as positive controls; cells incubated without conidia served as background controls. ROS release was measured at 5-min intervals for 45 min. All assays were performed in duplicate.

Measurement of fungal damage mediated by monocytes. The measurement of mitochondrial activity as a parameter for fungal damage has been established for adherent hyphae (24, 39). Conidia, however, are nonadherent and would be lost during the detergent lysis and washing steps. Therefore, we developed a novel approach for the release of phagocytized conidia by permeabilizing the cells with the pore-forming toxin streptolysin O (SLO). SLO treatment for 30 min leads to membrane permeabilization and killing of phagocytes (5). Fungal mitochondrial activity was unaffected by SLO and could be quantified without further washing steps in a menadione-augmented MTT test, as previously described (16, 17). Monocytes (3×10^5) were incubated with *A. fumigatus* conidia at an E/T ratio of 1:2 in minimal essential medium (MEM) supplemented with 10% normal human serum at 37°C with 5% CO_2 for 12 h. Incubations were carried out in flat-bottom microtiter plates (Nunc, Wiesbaden, FRG). The medium was then removed and replaced by 50 μl of MEM per well containing SLO at a final concentration of 20 μg/ml. After 30 min at 37°C, the monocytes were completely permeabilized and killed. Ten microliters of MEM containing 2.5 mg of MTT per ml and 0.5 mM menadione was added to each well, and after a further incubation at 37°C for 3 h, the MTT test was continued as described above. For comparison of strains, a damage index (DI) was calculated by applying the following equation: $DI = 1 - [(OD_{c+s} - OD_c)/OD_s]$, where OD_{c+s} is the optical density in wells containing conidia and cells, OD_c is the optical density in wells with cells only, and OD_s is the optical density in wells containing conidia only.

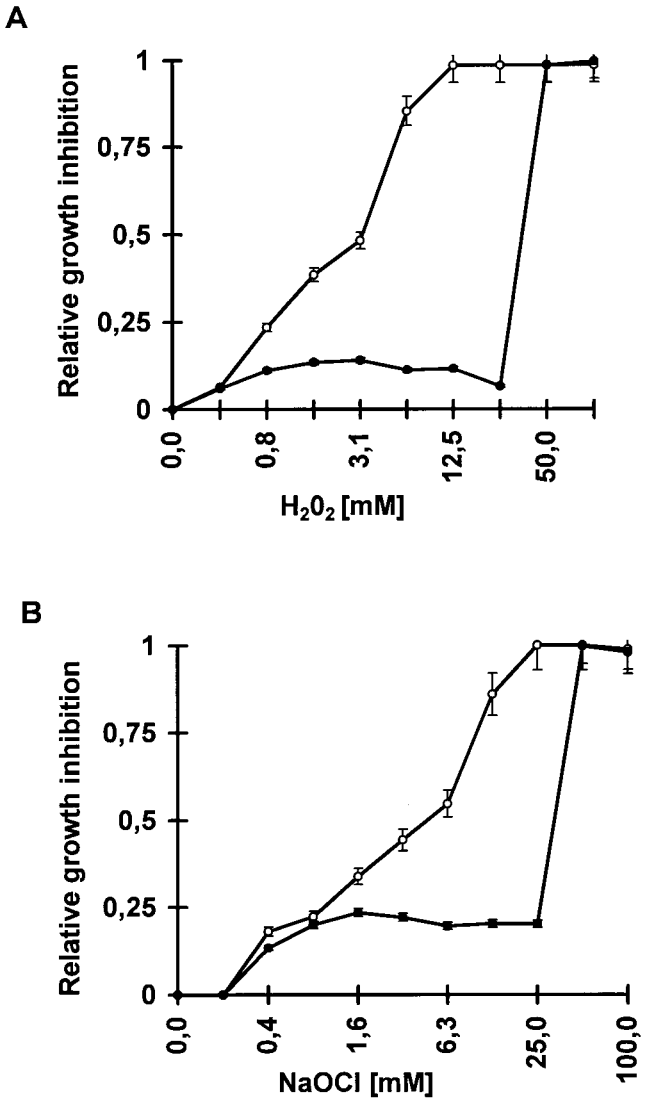

FIG. 2. Susceptibility of W (○) and WT (●) conidia to damage by oxidants. Conidia (10^5/well) were incubated at 30°C for 14 h with H_2O_2 (A) or NaOCl (B), and then an MTT test was performed. Wells incubated without oxidants served as a growth control; wells containing the oxidants only served as background controls. The calculated damage, given as relative growth inhibition, was plotted against the oxidant concentration. All assays were run in triplicate. Data are given as means and standard deviations.

In vivo testing of virulence. An intravenous murine animal model of invasive aspergillosis was employed according to standard procedures (12, 45, 50). *A. fumigatus* strains were grown on malt extract agar slopes (Difco, Detroit, Mich.) for 20 days at 28°C in the dark. Conidia were washed off with PBS containing 5 μl of Tween 20 (Sigma) per ml by using a Vortex mixer. This suspension was washed twice with PBS and further diluted with PBS to a final inoculum size of 2×10^7 conidia/ml, as determined by cell counting in a Neubauer chamber. Eight-week-old, specific-pathogen-free, male CFW1 mice (Hsd Win: CFW1; Harlan-Winkelmann, Paderborn, Germany) weighing 20 g were housed in groups of five in type two Macrolon cages (Ehret, Emmendingen, Germany). A group of 15 animals was infected with the *A. fumigatus* WT and W strains by injecting 0.2 ml of an inoculum suspension containing 4×10^6 conidia into the lateral tail vein with an 0.45- by 23-mm needle. In experiments with revertant strains WRV1 to -4, groups of five animals per strain were infected. The environmental temperature was kept constant at 23°C. Rat-mouse diet (Sniff, Soest, Germany) and water were provided ad libitum. Light-dark periods were adjusted to 12 h of light and 12 h of darkness daily. The acclimatization time before the experiment was 1 week. Animals were observed for mortality twice daily over a period of 9 days.

Statistical analysis. Statistical analysis was performed with Prism software (GraphPad Inc., San Diego, Calif.). Determination of statistical significance in the in vitro experiments was done by the two-tailed Student *t* test for unpaired samples. Mortality data in animal experiments were evaluated by Kaplan-Meyer plot analysis.

RESULTS

Isolation of an *A. fumigatus* mutant with altered conidia. The mutagenesis experiments described in Materials and Methods led to the isolation of a mutant strain which apparently lacked the ability to produce pigmented conidia and was therefore designated the white (W) strain. On AMM agar plates with glucose as the sole carbon source, at 28 and 37°C no difference between the growth rates of the W mutant strain and the WT was observed. Furthermore, the germination rates, as assessed by microscopy, and MTT conversion in submerged cultures were found to be the same for both strains.

Conidia of both the WT and the W mutant were analyzed by scanning electron microscopy (Fig. 1). WT conidia showed a rough surface covered with ornaments. In contrast, conidia of the W mutant had a smooth surface with hardly any ornaments on the conidial wall (Fig. 1). Further inspection of the whole conidiophore of the W mutant did not reveal additional major structural changes in its morphology compared with the WT (data not shown).

Fungal susceptibility to oxidants. Isolation of the W mutant strain enabled us to analyze whether the conidial pigment is important as protectant against ROS. Hence, viability of conidia after treatment with H_2O_2 and NaOCl was determined by the menadione-augmented MTT test (Fig. 2). For WT conidia, the IC_{50} of hydrogen peroxide was determined to be 40 mM, whereas for W conidia, the IC_{50} was 2 mM (Fig. 2A). A similar result was obtained when NaOCl was used: the IC_{50} for WT conidia was 35 mM, whereas that for W conidia was only 2.5 mM (Fig. 2B). Thus, nonpigmented W conidia were 10- to

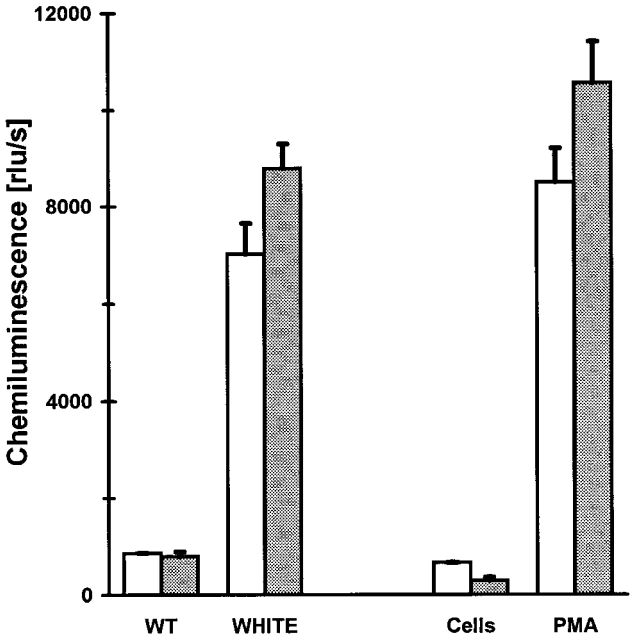

FIG. 3. Release of ROS caused by *A. fumigatus* conidia. PMN (□) or monocytes (■) (5×10^5/well) were incubated with 5×10^6 conidia of either the WT strain or strain W at 37°C for 20 min. Release of ROS was assessed by luminol-mediated chemiluminescence. Cells incubated without conidia served as a background control. Stimulation of cells with 10 nM PMA was employed as a positive control. Data represent means and standard deviations. All assays were run in duplicate. rlu, relative light units.

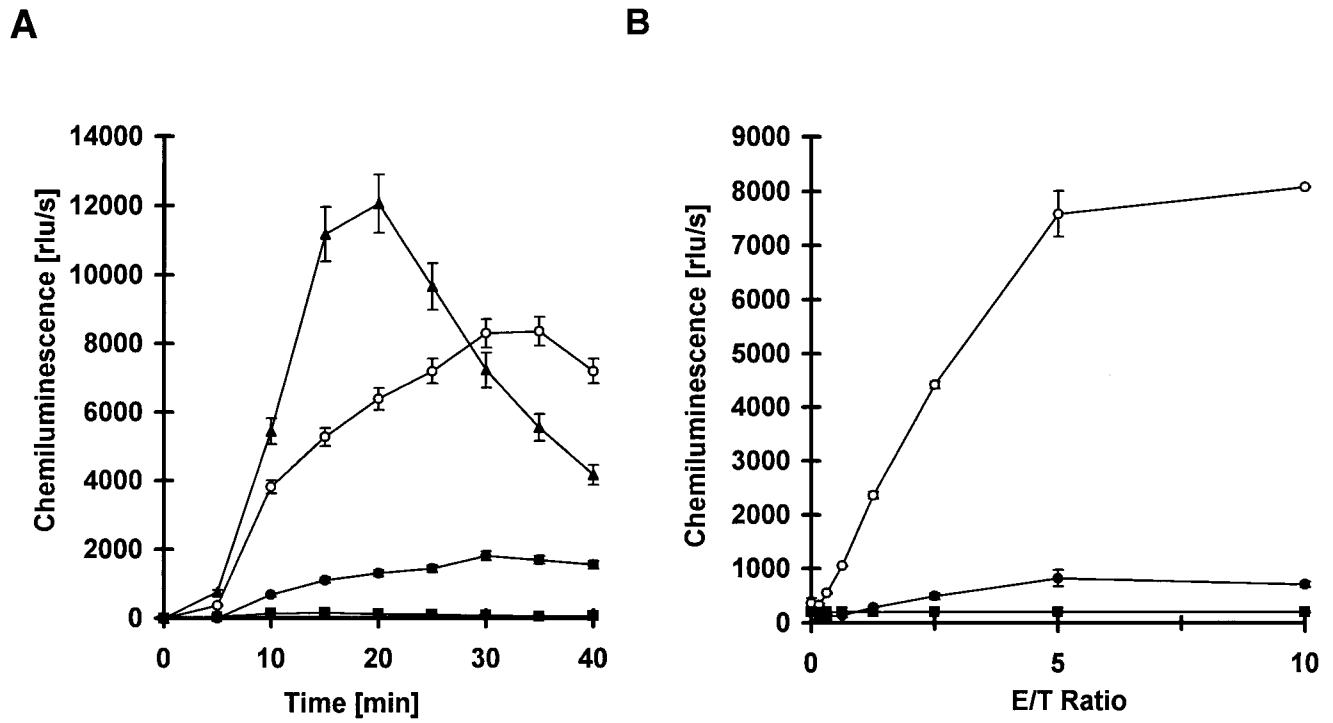

FIG. 4. Dose-response relationship and kinetics of ROS release by PMN induced by *A. fumigatus* conidia. PMN (5×10^5 per well) were incubated with W (○) or WT (●) conidia at 37°C for 40 min. PMA (10 nM) (▲) was used as a positive control; PMN alone (■) served as a background control. (A) Kinetics. All assays were run in triplicate ($n = 3$). (B) Dose-dependent curves. The E/T ratio was varied in a range of 1:0.16 to 1:10. A linear increase in ROS production by PMN was detected with W conidia up to an E/T ratio of 1:5. Further increases in the E/T ratio did not significantly augment the ROS response. WT conidia induced small amounts of ROS, and no clear dose dependency could be established. Data are given as means and standard deviations. rlu, relative light units.

12-fold more susceptible to oxidants than WT conidia, suggesting that the pigment plays an important role as a protectant against ROS.

ROS release by phagocytes caused by *A. fumigatus* WT and mutant conidia. Phagocytes are known to be a key component of the host defense against *Aspergillus*. To assess activation of phagocytes by both the W mutant and the WT, ROS release was measured after challenge with the respective conidia. Coincubation of phagocytes with W conidia resulted in a 10-fold increase in the amount of detectable ROS compared with that observed with WT conidia. This increase in ROS provoked by W conidia was comparable to that observed following stimulation of phagocytes with a standard inducer of the oxidative burst, 10 nM PMA (Fig. 3). WT conidia caused the release of only a small amount of ROS by PMN, and no ROS were measured after incubation of monocytes with these conidia (Fig. 3). The release of ROS was detectable after 5 min and reached a maximum after 15 to 20 min. After 30 min, ROS release decreased slowly (Fig. 4A) Incubation with WT conidia led to a small but constant ROS release during the whole incubation period (Fig. 4A). A dose-dependent release was observed for W conidia with E/T ratios of 1:0.3 to 1:5 (Fig. 4B).

Monocyte-mediated damage to outgrowing *A. fumigatus* mutant conidia. To evaluate potential differences between the WT and W mutant strains with regard to their susceptibility to intracellular anticonidial activity in monocytes, we assessed the damaging effects on conidia of both strains after ingestion and reisolation from human monocytes in vitro. Ingestion of mutant and WT conidia by monocytes was equally effective as seen by phase-contrast microscopy. However, marked differences in the growth-inhibitory effect of monocytic cells on the different conidia were observed. In the MTT test, inhibition of

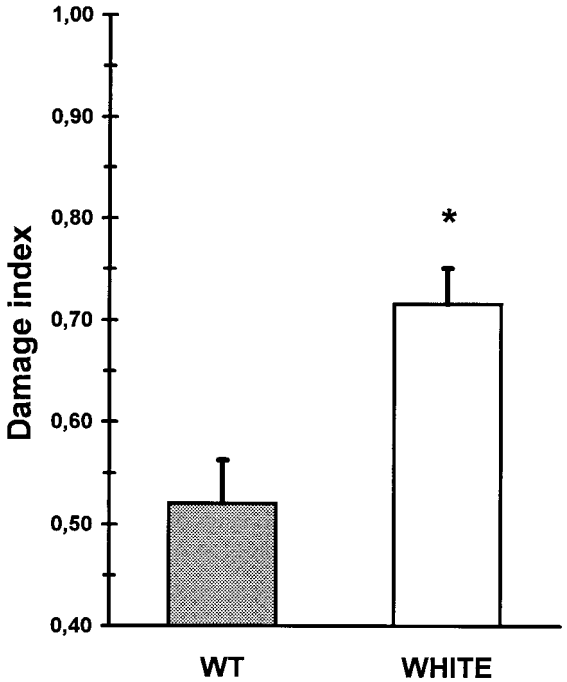

FIG. 5. In vitro damage of *A. fumigatus* conidia by monocytes. WT and W conidia were incubated with and without monocytes in MEM–10% normal human serum for 12 h at an E/T ratio of 1:1. For lysis of monocytes, wells were treated with SLO for 30 min and the MTT test was performed. The mean relative damage and standard deviation were calculated for each strain ($n = 3$). *, $P < 0.05$.

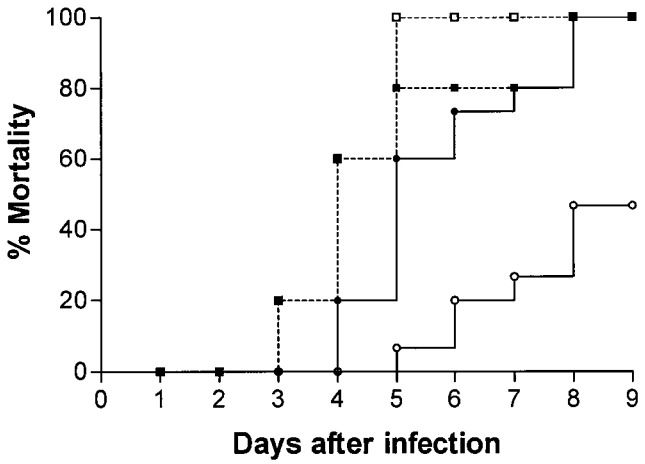

FIG. 6. Survival of mice after infection with *A. fumigatus* conidia. Animals were infected with conidia of *A. fumigatus* W (○), WT (●), WRV1 (■), and WRV2 (□) by lateral tail vein injection. For W and WT, 15 animals each were used, whereas for WRV strains, 5 animals each were infected. Survival was monitored for 9 days.

W conidia was 71%; this was significantly higher ($P < 0.05$) than that of WT conidia, which were inhibited by only 52% (Fig. 5). These findings suggest that the mutant strain was more effectively damaged by monocytes than the parental WT strain.

The W mutant displays reduced virulence in mice. To test the virulence of the W mutant, mice were injected with defined numbers of conidia and survival of animals was monitored over 9 days. No immediate reactions were observed directly after intravenous application of the conidia. As shown in Fig. 6, conidia of the WT strain caused death of all animals within 8 days. The virulence of conidia of the W strain was significantly reduced, to approximately 50%, as shown by the mortality of 7 of 15 for animals infected with W conidia (Fig. 6).

Isolation and analysis of revertants of the W mutant. The W mutant exhibited two phenotypes: lack of pigmentation and altered surface structure. These phenotypes could be due to several different mutations or a single mutation. In order to be able to elucidate the molecular basis of the reduced virulence of this strain, it was prerequisite to analyze how many mutations in the mutant caused these phenotypes. Because *A. fumigatus* as a deuteromycete lacks a sexual cycle and also because classical genetic methods based on parasexuality are poorly developed, a classical reversion analysis was performed. For this purpose, W conidia were subjected to UV mutagenesis, and among 4×10^4 colonies screened, four colonies that showed WT pigmentation, i.e., had reverted from the W to the green phenotype, were obtained. They were designated WRV1 (for W revertant 1) to WRV4. To rule out contamination with unrelated *Aspergillus* strains, revertants were analyzed by molecular typing according to the method of Aufauvre-Brown et al. (3). Chromosomal DNAs of the different *A. fumigatus* strains, control strains, and also related species were isolated and subjected to PCRs with oligonucleotide R151, as outlined in Materials and Methods. As shown in Fig. 7, the oligonucleotide allowed discrimination between the different *A. fumigatus* strains and also between *A. fumigatus* and the other analyzed *Aspergillus* species. The molecular typing demonstrated that all revertants, the W mutant, and the ancestor strain ATCC 46645 have the same molecular pattern, indicated by a DNA band of about 680 bp (Fig. 7).

Scanning electron microscopy revealed that reversion of pigmentation was paralleled by reversion of the smooth surface characteristic of W conidia to the rough WT conidial surface (Fig. 8). Since both phenotypes coreverted to the WT phenotype, this strongly suggests that both phenotypes are due to a single mutation. The IC_{50}s for W revertants exposed to hydrogen peroxide were the same as for WT conidia (data not shown). Furthermore, the ability of revertant conidia to stimulate the oxidative burst was also significantly lower than that of W conidia and reached the level of WT-induced ROS release (Fig. 9). The revertants were also tested in a murine animal model (Fig. 6). The mortality of animals infected with revertant conidia was 100% (five of five), as shown, for example, for WRV1 and WRV2 (Fig. 6). Similar results were obtained with conidia of WRV3 and WRV4 (data not shown). Taken together, these data demonstrated that both the host response against conidia of the revertants in vitro and the virulence of the conidia were restored and corresponded to those seen with WT conidia.

DISCUSSION

In most cases, inhalation of *A. fumigatus* conidia is the initial event of infection. Then, for establishing disease, the conidia need to germinate, which requires metabolic adaptation. They also must be able to withstand a possible attack by phagocytes. To gain insight into putative conidium-specific factors involved in virulence, an *A. fumigatus* mutant producing conidia that had lost conidial pigmentation was isolated. The conidia were tested for their ability to cause ROS production in human monocytes and PMN. Conidia of the WT strain led to only a small release of ROS, which agreed well with previous results reported by Levitz and Diamond (25). In contrast, however, conidia of the W mutant caused a strong ROS release by both cell types. Two explanations for this observation have to be considered. First, it could be due to the fact that conidia of both strains induced an oxidative burst to the same extent, but the produced ROS were scavenged by the pigment present in WT conidia and thus were not detected by chemiluminescence. For some fungal pigments the ability to scavenge ROS has been already shown (14, 47), and here this assumption was supported by the observation that W conidia were 10- to 12-

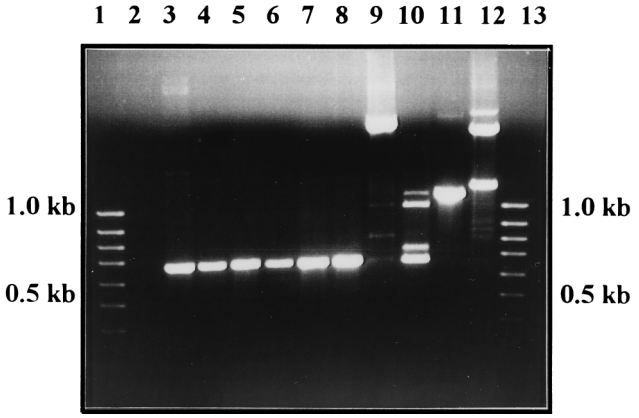

FIG. 7. DNA fragments amplified from genomic DNA samples from different *Aspergillus* strains. Numbers on the left and right indicate sizes of the components of a 100-bp DNA ladder. A 1.8% agarose gel was used. Lanes: 1, 100-bp ladder; 2, control reaction in which target DNA was omitted; 3, *A. fumigatus* revertant 1; 4, *A. fumigatus* revertant 2; 5, *A. fumigatus* revertant 3; 6, *A. fumigatus* revertant 4; 7, *A. fumigatus* WT strain ATCC 46645; 8, *A. fumigatus* W mutant; 9, *A. fumigatus* WT strain CBS 113.26; 10, *A. fumigatus* CBS 386.75 (albino); 11, *A. nidulans* R21; 12, *A. niger* N402; 13, 100-bp ladder.

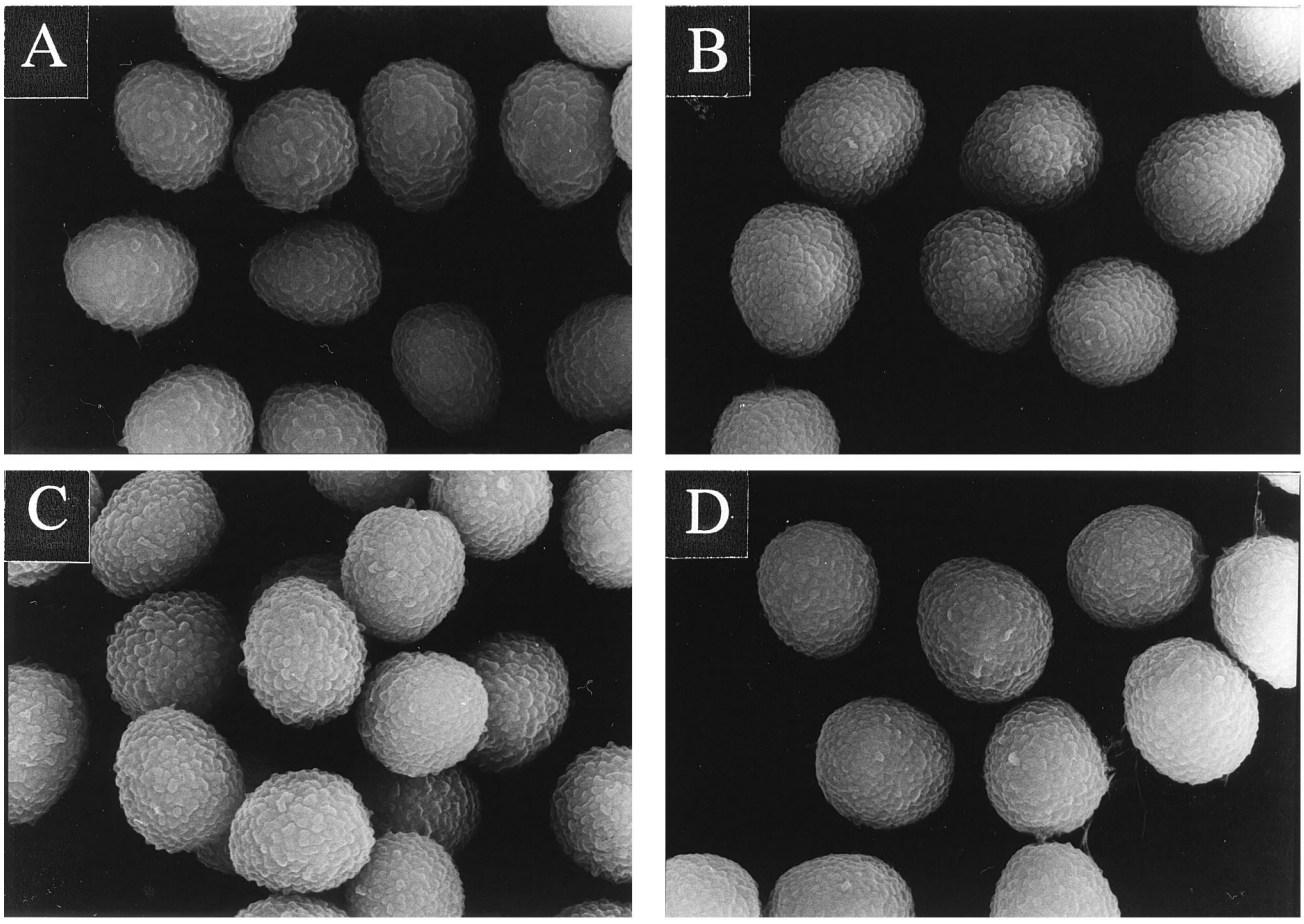

FIG. 8. Scanning electron microscopy photographs of conidia of revertant strains WRV1 (A), WRV2 (B), WRV3 (C), and WRV4 (D).

fold more sensitive to both hydrogen peroxide and NaOCl than WT conidia. This indicates that ROS scavenging also occurs in pigmented *A. fumigatus* conidia and thus confers relative protection against damage caused by ROS.

Alternatively, it is conceivable that W conidia were better recognized by phagocytes because the conidial surface of the W mutant differed markedly from that of the WT. This might result in a more efficient and stronger activation of phagocytes, which would explain the observed increase in production of ROS when the immune cells were challenged with W mutant conidia. As the two mechanisms, i.e., lack of quenching ability and activation by an altered conidial surface, are functionally independent, the observed difference in ROS release may well result from a combined effect of both mutant properties.

To quantify the damage of *A. fumigatus* conidia after their ingestion by monocytes, a modified MTT assay for conidial mitochondrial activity was developed. The results obtained with this test revealed that conidia of the W mutant were more efficiently damaged after phagocytosis than WT conidia. These in vitro findings obtained with monocytes correlated well with the results of in vivo experiments with immunocompetent mice. For the in vivo studies, an intravenous murine model of invasive aspergillosis was chosen because it is well standardized and reproducible. This animal model obviated the induction of immunosuppression with the risk of concomitant bacterial infections and allowed an exact definition of the infection dose (50). It is worth noting that the systemic intravenous applica-

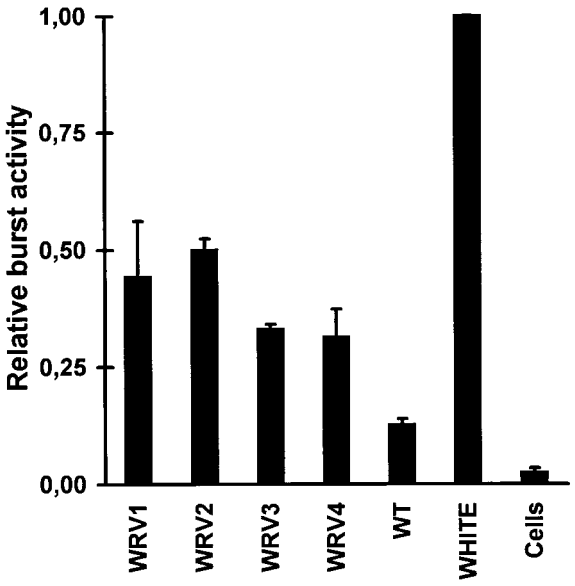

FIG. 9. Relative induction of oxidative burst by W revertant conidia. Cells (5×10^5 per well) were incubated with 10^6 conidia of revertant strains (WRV1 to WRV4), the W mutant, or the WT at 37°C for 20 min. Release of ROS was assessed by luminol-mediated chemiluminescence. Cells incubated without conidia served as a negative control. The value obtained with W conidia was set at 1.00. The relative values given for conidia of each strain represent the means and standard deviations for two independent experiments.

tion leads to typical granulomatous foci with macrophages and neutrophils as predominant cell types (22, 50), and results comparable to those with the inhalation model have been obtained in various pharmaceutical studies (11). In the intravenous animal model of invasive aspergillosis used here, a significant reduction of virulence was observed with the W mutant. Hence, these in vivo studies supported our model based on the in vitro data; i.e., loss of pigmentation renders conidia more susceptible to ROS, and/or W conidia were better recognized by phagocytes.

The mutant strain described here was generated from a defined parental American Type Culture Collection strain by UV mutagenesis. An important genetic question concerned the number of mutations present in the W mutant and causing the observed phenotypes. A classical reversion analysis of the W mutant led to the finding that, most likely, a single mutation caused both phenotypes of the conidia, i.e., lack of conidial pigmentation and smooth cell surface structure.

Compared with W conidia, all of the isolated revertant conidia also had a reduced ability to induce an oxidative burst in both PMN and monocytes. The amount of ROS released upon challenge with revertant conidia was comparable to that seen with WT conidia (Fig. 9). In addition, when the revertant conidia were tested for virulence in the mouse model, the same mortality rate as observed after infection with WT conidia was attained. Taken together, these data show that all phenotypes attributed to W conidia, i.e., altered conidial surface, amount of ROS release, damage by monocytes, and reduced virulence in a murine animal model, coreverted in all four revertants which had regained the ability to produce green spores. This finding strongly suggests that the *A. fumigatus* mutant described here carried a single mutation which caused all of the observed phenotypes. Hence, we propose that this feature of WT conidia which is lost in W conidia, apparently by a single mutation, contributes to pathogenicity of *A. fumigatus.*

Whether this mutation directly interfered with pigment synthesis remains to be elucidated. It is also conceivable that a factor required for correct processing or embbeding of pigment into the cell wall was mutated, thus resulting in altered surface structures of W conidia. Previously, a spontaneous white-spored mutant strain of *A. fumigatus* was isolated (6). The mutation was complemented by transforming the strain with a plasmid containing the *wA* gene from *A. nidulans*, suggesting that the mutant carries a defective *wA* gene (6). At present, the molecular basis of the W mutant characterized here with regard to virulence has not been analyzed. The cloning of the corresponding W gene, however, which is currently in progress, will help to answer questions related to its structure and function.

ACKNOWLEDGMENTS

August Böck is gratefully acknowledged for much helpful advice and discussion. We thank M. Husmann and S. Gatermann for helpful discussion and I. Dürr and A. Jahn for excellent technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft (grant Br 1130/5-1 to A.A.B.) and in part by Sonderforschungsbereich 369 (to G.W.).

REFERENCES

1. **Arruda, L. K., B. J. Mann, and M. D. Chapman.** 1992. Selective expression of a major allergen and cytotoxin, Asp f I, in *Aspergillus fumigatus*. Implications for the immunopathogenesis of Aspergillus-related diseases. J. Immunol. **149:**3354–3359.
2. **Arruda, L. K., T. A. Platts Mills, J. W. Fox, and M. D. Chapman.** 1990. *Aspergillus fumigatus* allergen I, a major IgE-binding protein, is a member of the mitogillin family of cytotoxins. J. Exp. Med. **172:**1529–1532.
3. **Aufauvre-Brown, A., J. Cohen, and D. W. Holden.** 1992. Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J. Clin. Microbiol. **30:**2991–2993.
4. **Babior, B. M.** 1978. Oxygen-dependent microbial killing by phagocytes. N. Engl. J. Med. **298:**659–668.
5. **Bhakdi, S., U. Weller, I. Walev, E. Martin, D. Jonas, and M. Palmer.** 1993. A guide to the use of pore-forming toxins for controlled permeabilization of cell membranes. Med. Microbiol. Immunol. **182:**167–175.
6. **Borgia, P. T., C. L. Dodge, L. E. Eagleton, and T. H. Adams.** 1994. Bidirectional gene transfer between *Aspergillus fumigatus* and *Aspergillus nidulans*. FEMS Microbiol. Lett. **122:**227–232.
7. **Bouchara, J. P., G. Tronchin, G. Larcher, and D. Chabasse.** 1995. The search for virulence determinants in *Aspergillus fumigatus*. Trends Microbiol. **3:**327–330.
8. **Brakhage, A. A., and J. Van den Brulle.** 1995. Use of reporter genes to identify recessive *trans*-acting mutations specifically involved in the regulation of *Aspergillus nidulans* penicillin biosynthesis genes. J. Bacteriol. **177:** 2781–2788.
9. **Coulot, P., J. P. Bouchara, G. Renier, V. Annaix, C. Planchenault, G. Tronchin, and D. Chabasse.** 1994. Specific interaction of *Aspergillus fumigatus* with fibrinogen and its role in cell adhesion. Infect. Immun. **62:**2169–2177.
10. **Denning, D. W.** 1994. Invasive aspergillosis in immunocompromised patients. Curr. Opin. Infect. Dis. **7:**456–462.
11. **Denning, D. W., L. Hall, M. Jackson, and S. Hollis.** 1995. Efficacy of D0870 compared with those of itraconazole and amphotericin B in two murine models of invasive aspergillosis. Antimicrob. Agents Chemother. **39:**1809–1814.
12. **Graybill, J. R., and J. Ahrens.** 1985. Itraconazole treatment of murine aspergillosis. Sabouraudia **23:**219–223.
13. **Holden, D. W., C. M. Tang, and J. M. Smith.** 1994. Molecular genetics of Aspergillus pathogenicity. Antonie Leeuwenhoek **65:**251–255.
14. **Jacobson, E. S., E. Hove, and H. S. Emery.** 1995. Antioxidant function of melanin in black fungi. Infect. Immun. **63:**4944–4945.
15. **Jacobson, E. S., and S. B. Tinnell.** 1993. Antioxidant function of fungal melanin. J. Bacteriol. **175:**7102–7104.
16. **Jahn, B., E. Martin, A. Stueben, and S. Bhakdi.** 1995. Susceptibility testing of *Candida albicans* and *Aspergillus* species by a simple microtiter menadione-augmented 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. J. Clin. Microbiol. **33:**661–667.
17. **Jahn, B., A. Stueben, and S. Bhakdi.** 1996. Colorimetric susceptibility testing for *Aspergillus fumigatus*: comparison of menadione-augmented 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide and alamar blue tests. J. Clin. Microbiol. **34:**2039–2041.
18. **Kolattukudy, P. E., J. D. Lee, L. M. Rogers, P. Zimmerman, S. Ceselski, B. Fox, B. Stein, and E. A. Copelan.** 1993. Evidence for possible involvement of an elastolytic serine protease in aspergillosis. Infect. Immun. **61:**2357–2368.
19. **Kramer, M. R., S. E. Marshall, V. A. Starnes, P. Gamberg, Z. Amitai, and J. Theodore.** 1993. Infectious complications in heart-lung transplantation. Analysis of 200 episodes. Arch. Intern. Med. **153:**2010–2016.
20. **Kwon Chung, K. J., I. Polacheck, and T. J. Popkin.** 1982. Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. J. Bacteriol. **150:**1414–1421.
21. **Kwon Chung, K. J., and J. C. Rhodes.** 1986. Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. Infect. Immun. **51:**218–223.
22. **Kwon-Chung, K. J., and J. E. Bennett.** 1992. Aspergillosis, p. 201–247. *In* K. J. Kwon-Chung and J. E. Bennett (ed.), Medical mycology. Lea and Febiger, Philadelphia, Pa.
23. **Lamy, B., M. Moutaouakil, J. P. Latge, and J. Davies.** 1991. Secretion of a potential virulence factor, a fungal ribonucleotoxin, during human aspergillosis infections. Mol. Microbiol. **5:**1811–1815.
24. **Levitz, S. M., and R. D. Diamond.** 1985. A rapid colorimetric assay of fungal viability with the tetrazolium salt MTT. J. Infect. Dis. **152:**938–945.
25. **Levitz, S. M., and R. D. Diamond.** 1985. Mechanisms of resistance of *Aspergillus fumigatus* conidia to killing by neutrophils in vitro. J. Infect. Dis. **152:**33–42.
26. **Litzka, O., K. Bergh, and A. A. Brakhage.** 1995. Analysis of the regulation of the *Aspergillus nidulans* penicillin biosynthesis gene AAT (PENDE), which encodes acyl coenzyme A:6-aminopenicillanic acid acyltransferase. Mol. Gen. Genet. **249:**557–569.
27. **Martin, E., and S. Bhakdi.** 1991. Quantitative analysis of opsonophagocytosis and of killing of *Candida albicans* by human peripheral blood leukocytes by using flow cytometry. J. Clin. Microbiol. **29:**2013–2023.
28. **Martin, E., A. Stuben, A. Gorz, U. Weller, and S. Bhakdi.** 1994. Novel aspect of amphotericin B action: accumulation in human monocytes potentiates killing of phagocytosed *Candida albicans*. Antimicrob. Agents Chemother. **38:**13–22.
29. **McWhinney, P. H., C. C. Kibbler, M. D. Hamon, O. P. Smith, L. Gandhi, L. A. Berger, R. K. Walesby, A. V. Hoffbrand, and H. G. Prentice.** 1993. Progress in the diagnosis and management of aspergillosis in bone marrow transplantation: 13 years' experience. Clin. Infect. Dis. **17:**397–404.
30. **Monod, M., S. Paris, D. Sanglard, K. Jaton Ogay, J. Bille, and J. P. Latge.** 1993. Isolation and characterization of a secreted metalloprotease of *As-*

pergillus fumigatus. Infect. Immun. **61:**4099–4104.
31. **Monod, M., S. Paris, J. Sarfati, K. Jaton Ogay, P. Ave, and J. P. Latge.** 1993. Virulence of alkaline protease-deficient mutants of *Aspergillus fumigatus*. FEMS Microbiol. Lett. **80:**39–46.
32. **Monod, M., G. Togni, L. Rahalison, and E. Frenk.** 1991. Isolation and characterisation of an extracellular alkaline protease of *Aspergillus fumigatus*. J. Med. Microbiol. **35:**23–28.
33. **Moser, M., R. Crameri, G. Menz, T. Schneider, T. Dudler, C. Virchow, M. Gmachl, K. Blaser, and M. Suter.** 1992. Cloning and expression of recombinant *Aspergillus fumigatus* allergen I/a (rAsp f I/a) with IgE binding and type I skin test activity. J. Immunol. **149:**454–460.
34. **Musial, C. E., F. R. Cockerill, and G. D. Roberts.** 1988. Fungal infections of the immunocompromised host: clinical and laboratory aspects. Clin. Microbiol. Rev. **1:**349–364.
35. **Paris, S., M. Monod, M. Diaquin, B. Lamy, L. K. Arruda, P. J. Punt, and J. P. Latge.** 1993. A transformant of *Aspergillus fumigatus* deficient in the antigenic cytotoxin ASPFI. FEMS Microbiol. Lett. **111:**31–36.
36. **Raeder, U., and P. Broder.** 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. **1:**17–20.
37. **Reichard, U., S. Buttner, H. Eiffert, F. Staib, and R. Ruchel.** 1990. Purification and characterisation of an extracellular serine proteinase from *Aspergillus fumigatus* and its detection in tissue. J. Med. Microbiol. **33:**243–251.
38. **Reichard, U., M. Monod, and R. Ruchel.** 1995. Molecular cloning and sequencing of the gene encoding an extracellular aspartic proteinase from *Aspergillus fumigatus*. FEMS Microbiol. Lett. **130:**69–74.
39. **Roilides, E., A. Holmes, C. Blake, D. Venzon, P. A. Pizzo, and T. J. Walsh.** 1994. Antifungal activity of elutriated human monocytes against *Aspergillus fumigatus* hyphae: Enhancement by granulocyte-macrophage colony-stimulating factor and interferon-gamma. J. Infect. Dis. **170:**894–899.
40. **Roilides, E., K. Uhlig, D. Venzon, P. A. Pizzo, and T. J. Walsh.** 1993. Prevention of corticosteroid-induced suppression of human polymorphonuclear leukocyte-induced damage of *Aspergillus fumigatus* hyphae by granulocyte colony-stimulating factor and gamma interferon. Infect. Immun. **61:**4870–4877.
41. **Rowlands, R. T., and G. Turner.** 1973. Nuclear and extranuclear inheritance of oligomycin resistance in *Aspergillus nidulans*. Mol. Gen. Genet. **126:**201–216.
42. **Smith, J. M., C. M. Tang, S. Van Noorden, and D. W. Holden.** 1994. Virulence of *Aspergillus fumigatus* double mutants lacking restriction and an alkaline protease in a low-dose model of invasive pulmonary aspergillosis. Infect. Immun. **62:**5247–5254.
43. **Stephan, J. L., V. Vlekova, F. Le Deist, S. Blanche, J. Donadieu, G. De Saint Basile, A. Durandy, C. Griscelli, and A. Fischer.** 1993. Severe combined immunodeficiency: a retrospective single-center study of clinical presentation and outcome in 117 patients. J. Pediatr. **123:**564–572.
44. **Tronchin, G., J. P. Bouchara, G. Larcher, J. C. Lissitzky, and D. Chabasse.** 1993. Interaction between *Aspergillus fumigatus* and basement membrane laminin: binding and substrate degradation. Biol. Cell **77:**201–208.
45. **Van Cutsem, J., F. Van Gerven, and P. A. Janssen.** 1987. Activity of orally, topically, and parenterally administered itraconazole in the treatment of superficial and deep mycoses: animal models. Rev. Infect. Dis. **9**(Suppl. 1)**:** S15–S32.
46. **Wang, Y., P. Aisen, and A. Casadevall.** 1995. *Cryptococcus neoformans* melanin and virulence: mechanism of action. Infect. Immun. **63:**3131–3136.
47. **Wang, Y., and A. Casadevall.** 1994. Susceptibility of melanized and nonmelanized *Cryptococcus neoformans* to nitrogen- and oxygen-derived oxidants. Infect. Immun. **62:**3004–3007.
48. **Washburn, R. G., J. I. Gallin, and J. E. Bennett.** 1987. Oxidative killing of *Aspergillus fumigatus* proceeds by parallel myeloperoxidase-dependent and -independent pathways. Infect. Immun. **55:**2088–2092.
49. **Watson, F., J. Robinson, and S. W. Edwards.** 1991. Protein kinase C-dependent and -independent activation of the NADPH oxidase of human neutrophils. J. Biol. Chem. **266:**7432–7439.
50. **Yamaguchi, H.** 1987. Opportunistic fungal infections, p. 101–158. *In* M. Miyaji (ed.), Animal models in medical mycology. CRC Press, Boca Raton, Fla.
51. **Zuger, A., E. Louie, R. S. Holzman, M. S. Simberkoff, and J. J. Rahal.** 1986. Cryptococcal disease in patients with the acquired immunodeficiency syndrome. Diagnostic features and outcome of treatment. Ann. Intern. Med. **104:**234–240.

Editor: T. R. Kozel