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The Alkaline Protease of *Aspergillus fumigatus* Is Not a Virulence Determinant in Two Murine Models of Invasive Pulmonary Aspergillosis

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Little is known of the pathophysiology of invasive pulmonary aspergillosis (IPA), an opportunistic fungal infection usually caused by *Aspergillus fumigatus*. It has been suggested that the ability of the fungus to degrade elastin may aid its invasion and growth in lung tissue. We have described previously the construction of a strain of *A. fumigatus* in which the gene encoding an alkaline protease, AFAlp, had been disrupted (C. M. Tang, J. Cohen, and D. W. Holden, Mol. Microbiol. 6:1663–1671, 1992); this mutant is deficient in extracellular proteolytic and elastinolytic activity over a broad pH range. In this study, we compared the pathogenicity of this and another AFAlp disruptant with their isogenic, elastase-producing parental strains in two murine models of IPA. In both models, animals were inoculated via the respiratory tract. In the first model, the inoculum was delivered as airborne conidia and animals developed signs of respiratory distress within 2 to 4 days. In the second model, conidia were administered intranasally as a suspension and the disease developed over a 2-week period. No difference was observed between the wild-type and AFAlp disruptants in terms of mortality, and elastin breakdown was detected in lung tissue from animals inoculated with all four strains. We conclude that AFAlp is not a virulence determinant in these models of IPA.

Aspergillus fumigatus is an important fungal pathogen of the immunocompromised host that causes a life-threatening respiratory infection, invasive pulmonary aspergillosis (IPA) (2). The disease is acquired by the inhalation of airborne conidia. Following germination, hyphae invade through the walls of bronchi and smaller airways into the lung parenchyma, resulting in a necrotizing bronchopneumonia. The fungus can spread to invade the arterial tree, causing vascular occlusion with hemorrhagic infarction and dissemination to other organs. As the infection progresses, necrosis of the pulmonary parenchyma may lead to cavitation.

Little is known about the virulence determinants of *A. fumigatus*, though it has been suggested that the production of extracellular proteases, such as elastase(s), by the fungus may be important in allowing it to invade and grow in lung tissue. There is indirect evidence to support this hypothesis. Rhodes et al. (20) showed that while only 13 of 27 isolates of *Aspergillus* spp. recovered from the environment were elastase producers, all 11 clinical isolates produced the enzyme. In a study by Kothary et al. (11), environmental isolates of *A. fumigatus* were assessed for their ability to produce elastase both by a plate assay and in liquid culture. Isolates which produced elastase caused higher mortality and tissue invasion than elastase nonproducers when tested in a murine model of IPA. Those authors proposed that fungal elastase might be important in the process of tissue invasion by *A. fumigatus*. However, the strains tested in their study were not isogenic, and therefore, the correlation between elastase production and pathogenicity may have been due to other factors associated with the elastase-producing strains. Also, it is noteworthy that the same group of workers has since reported that isolates designated "elastase nonproducers" in their original study were in fact elastase producers when assayed by a different method (8). Furthermore, in sections of lung tissue from patients with IPA, Denning et al. (6) found no evidence of elastinolysis in the walls of arteries which had been invaded by the fungus.

The biochemical basis of extracellular elastinolysis by *A. fumigatus* has been investigated by several groups (8, 17, 19), who have all identified an alkaline protease with elastinolytic activity and a molecular mass (M_r) of 32 to 33 kDa. Reichard et al. (19) characterized an alkaline protease, AFAlp, as having an estimated M_r of 32 kDa and a pI of 7.9. The sequence of amino acids at the N terminus of the mature protein indicated that it is closely related to an alkaline protease of *Aspergillus oryzae* (25) that is a member of the subtilisin family. Monod et al. (17) purified from *A. fumigatus* an alkaline protease with an M_r of 33 kDa; subsequent identification and sequencing of both genomic and cDNA clones encoding this protease (10) showed that it is identical to AFAlp. More recently, Frosco et al. (8) reported an alkaline protease with an M_r of 32 kDa and a pI of 8.8. They concluded that this was not AFAlp because of discrepancies in the isoelectric point and inhibitor profile of their enzyme; however, they used a different substrate to assay the residual activity of their enzyme after the addition of inhibitors.

We used gene disruption to inactivate the AFAlp gene in an elastase-producing clinical isolate of *A. fumigatus*. The mutant is deficient in extracellular elastinolytic and proteolytic activities over the pH range 6 to 10.4 and lacks an extracellular protein of 33 kDa (24). This work showed that in this isolate at least, there is only one alkaline protease with elastinolytic activity. In this study, we have compared the pathogenicity of isogenic wild-type and AFAlp disruptant strains of *A. fumigatus* in two murine models of IPA and

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were unable to show a difference between them in terms of disease phenotype.

MATERIALS AND METHODS

Fungal strains and AFAlp gene disruption. Strain 234 was cultured from bronchoalveolar lavage fluid from a patient with IPA at the Hammersmith Hospital, London, United Kingdom. Strain 234d was derived from 234 by single-gene disruption of the AFAlp gene (24). Strain 234e was derived from strain 234 by single-copy ectopic integration of the disruption vector pID21, which contains the hygromycin phosphotransferase gene of *Escherichia coli* under the control of the *trpC* promoter of *Aspergillus nidulans* (4). Strain 237 was cultured from open-lung biopsy material from a patient with IPA at Hope Hospital, Manchester, United Kingdom (gift from M. Keaney). Transformation of this strain with pID21 gave rise to an AFAlp gene disruptant (strain 237d) and a single-copy ectopic integrant (strain 237e).

DNA extraction, PCRs, and RAPD analysis. Small-scale DNA extraction from fungal isolates was performed as previously described (24). Positive displacement pipettes were used throughout. Primer alp11 (5′-AGCACCGACTA CATCTAC-3′) was derived from the sequence of the AFAlp gene corresponding to amino acids 143 to 148 (10) upstream of the *Kpn*I-*Sal*I fragment in pID21 (Fig. 1). Oligonucleotide pbr1 (5′-TTCCTAATGCAGGAGTCG-3′) was based on pBR322 sequences in pID21 (4). When these primers were used, a 705-bp product was expected following amplification of DNA from an AFAlp disruptant. No product was expected when DNA from a parental or ectopic transformant strain of *A. fumigatus* was employed as target. Primers rib1 (5′-GCGAAGCTTACGTCCAAGATGGTTGCA-3′) and rib2 (5′-GCGAAGCTTACACCTTGTTGGGATAAG-3′) were based on sequences from the restrictocin gene of *A. fumigatus* (14) and included *Hin*dIII restriction enzyme recognition sites. Primers rib1 and rib2 were incorporated in polymerase chain reactions (PCRs) as internal controls; the expected size of product when these two primers were used was 547 bp. Primers were synthesized on an oligonucleotide synthesizer (Applied Biosystems, model 380B) in the Department of Virology, Royal Postgraduate Medical School. PCRs were carried out in a 50-μl volume containing 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-Cl (pH 8.0) with 10 ng of DNA as target; 250 μM each dATP, dCTP, dTTP, and dGTP; 100 pmol of each primer; and 1.25 U of Amplitaq (Perkin Elmer Cetus, Norwalk, Conn.). Thermal cycling conditions were 32 cycles of 94°C for 45 s, 56°C for 30 s, and 72°C for 2 min. Amplification products were separated on a 1.2% agarose gel, and visualized by UV transillumination after staining with ethidium bromide.

Random amplified polymorphic DNA (RAPD) analysis was carried out as described previously (1). Primer R151 (5′-TTCCGCGGGC-3′) was used to generate RAPD markers. Products were analyzed on a 1.2% agarose gel.

Infection models. (i) Inoculation by inhalation of airborne conidia. Inhalation chambers were made as described previously (18). In brief, they consisted of 1-liter flasks, each with eight side arms (internal diameter, 23 mm) for the mice and a further side arm for air sampling. Fungal strains were grown on Sabouraud dextrose agar slants in 100-ml bottles for 3 days at 37°C. Conidia were harvested by washing the slants with 0.1% Tween 80, centrifuged at 3,000 × *g* for 15 min, and finally suspended in saline. The concentration of conidia was determined by using a hemocytometer. Sabouraud dextrose agar (150 ml) at the base of the inhalation chamber was inoculated either with 3×10^7 conidia in 1 ml of saline or with saline alone and incubated at 37°C for 3 days. White male mice (strain CD1; Charles River Breeders, Kent, United Kingdom) weighing 14 to 17 g were immunosuppressed with cortisone acetate (Sigma, Poole, United Kingdom) (2 mg in 0.2 ml of 0.1% Tween 80) given subcutaneously on days −2 and 0. The animals were kept in cages with filter tops and given sterile food and sterile bedding. Tetracycline (0.5 mg/ml; Sigma) was added to the drinking water. On day 0, mice were introduced into the side arms of the chambers, and the conidia were dispersed by pumping 100 ml of air over the fungal colony at the base of the flask.

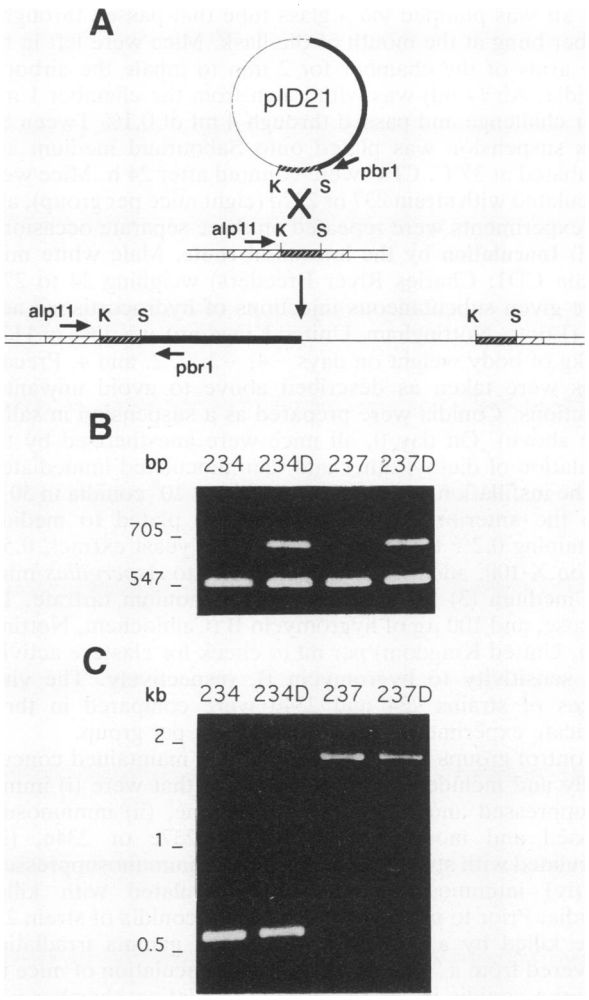

FIG. 1. (A) Disruption of the AFAlp gene by pID21. Hatched area represents the coding region of the AFAlp gene; the 504-bp *Kpn*I (K)-*Sal*I (S) fragment is represented by heavy hatching. Locations of the primers (alp11 and pbr1) used to identify AFAlp disruptants are shown. (B) Representative results of PCRs. Primers alp11 and pbr1 give rise to a 757-bp product when DNA from an AFAlp disruptant is employed as target; primers rib1 and rib2 were included as internal controls for amplification and give rise to a 504-bp product. The target DNA included in the reactions is indicated above each lane, and sizes of the products are shown. (C) DNA fragments amplified from genomic DNA with primer 151 (1). The target DNA employed in the reactions is indicated above each lane, and sizes of components of a 1-kb ladder (GIBCO-BRL) are shown.

The air was pumped via a glass tube that passed through a rubber bung at the mouth of the flask. Mice were left in the side arms of the chamber for 2 min to inhale the airborne conidia. Air (4 ml) was withdrawn from the chamber 1 min after challenge and passed through 4 ml of 0.1% Tween 80. This suspension was plated onto Sabouraud medium and incubated at 37°C. CFU were counted after 24 h. Mice were inoculated with strain 237 or 237d (eight mice per group), and the experiments were repeated on three separate occasions.

(ii) Inoculation by the intranasal route. Male white mice (strain CD1; Charles River Breeders) weighing 24 to 27 g were given subcutaneous injections of hydrocortisone acetate (Boots, Nottingham, United Kingdom) at a dose of 112.5 mg/kg of body weight on days −4, −2, 0, 2, and 4. Precautions were taken as described above to avoid unwanted infections. Conidia were prepared as a suspension in saline (see above). On day 0, all mice were anesthetized by the inhalation of diethyl ether and then inoculated immediately by the instillation of a suspension of 8×10^5 conidia in 30 μl into the anterior nares. Inocula were plated to medium containing 0.2% elastin (Sigma), 0.01% yeast extract, 0.5% Triton X-100, and 1.5% agar (8) and onto *Aspergillus* minimal medium (3) containing 5 mM ammonium tartrate, 1% glucose, and 100 μg of hygromycin B (Calbiochem, Nottingham, United Kingdom) per ml to check for elastase activity and sensitivity to hygromycin B, respectively. The virulences of strains 234 and 234d were compared in three replicate experiments involving 10 mice per group.

Control groups for both models were maintained concurrently and included 10 mice per group that were (i) immunosuppressed and inoculated with saline, (ii) immunosuppressed and inoculated with strain 237e or 234e, (iii) inoculated with strain 237 or 234 but not immunosuppressed, or (iv) immunosuppressed and inoculated with killed conidia. Prior to intranasal inoculation, conidia of strain 234 were killed by a dose of 5,600 Gy of gamma irradiation delivered from a ^{65}Co source. Before inoculation of mice by airborne conidia of strain 237, the inhalation chamber was incubated at 65°C for 3 h. In both cases, this resulted in a survival rate of $<3 \times 10^{-7}$.

All mice were observed for 15 days after inoculation, and mortality was recorded. Carbon dioxide was used to induce narcosis in distressed animals; the lungs were dissected and weighed. The left lung was homogenized in 500 μl of saline, plated to Sabouraud medium, and then incubated at 37°C for 48 h, after which the CFU were counted and the CFU per gram of lung tissue were calculated. Fungi recovered from the mice were tested for extracellular elastinolytic activity by growth on medium containing elastin (see above). The right lung was fixed in 10% buffered formol-saline for histological examination. Sections were stained with hematoxylin and eosin, Grocott's methenamine silver nitrate, and elastic van Gieson by standard techniques.

Statistical methods. The survival of mice was analyzed by the log rank method.

RESULTS

AFAlp gene disruption in strain 237, PCRs, and RAPD analysis. To identify AFAlp disruptants, PCRs were performed with primers alp11 and pbr1 on DNA from transformants. Following transformation of strain 237 with pID21, DNA from one of four hygromycin-resistant colonies gave rise to the 705-bp product expected from an AFAlp disruptant. Southern analysis was used to confirm that pID21 had integrated as a single copy and only at the AFAlp locus and to identify a single-copy ectopic integrant, 237e (data not shown). When 237d was examined on medium containing elastin as the sole nitrogen source, no evidence of residual extracellular elastinolytic activity was detected, which agrees with our previous results with disruption of the AFAlp gene in strain 234 (24). DNA samples from all isolates recovered from animals inoculated with AFAlp disruptant strains gave rise to amplification product of the expected size by the PCR assay. Because of the risk of contamination by airborne conidia, we used RAPD analysis to differentiate among strain 234, strain 237, and the derivatives of these strains. Representative results of PCRs and RAPD analysis are shown in Fig. 1.

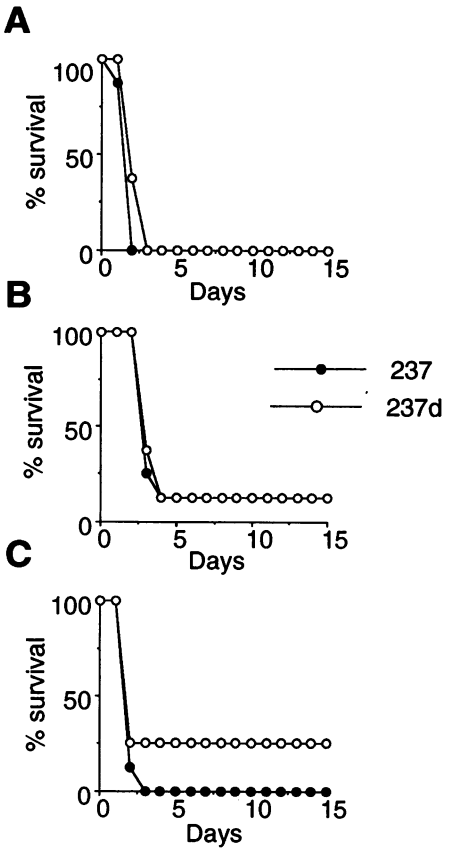

FIG. 2. Survival curves of mice inoculated by the inhalation of airborne conidia of strains 237 and 237d. Each experiment (A, B, and C) consisted of eight mice per strain.

Virulence in mice. (i) Inhalation model. Mice receiving immunosuppression alone, inoculation with 237 alone, or immunosuppression and inoculation with killed conidia remained healthy throughout the course of the experiments (data not shown). The survival of immunosuppressed mice following inoculation with either 237 or 237d is given in Fig. 2. Log rank analysis of survival curves from the experiments assessed both individually and as pooled data showed no significant difference in mortality between animals inoculated with each of the fungal strains. The number of CFU recovered from 4 ml of air withdrawn from the inhalation chambers 1 min after challenge with the fungus were as follows for strains 237 and 237d, respectively: experiment A, 5.36×10^5 and 5.68×10^5; experiment B, 4.4×10^5 and 3.6×10^5; and experiment C, 1.64×10^5 and 1.24×10^5. Fungal

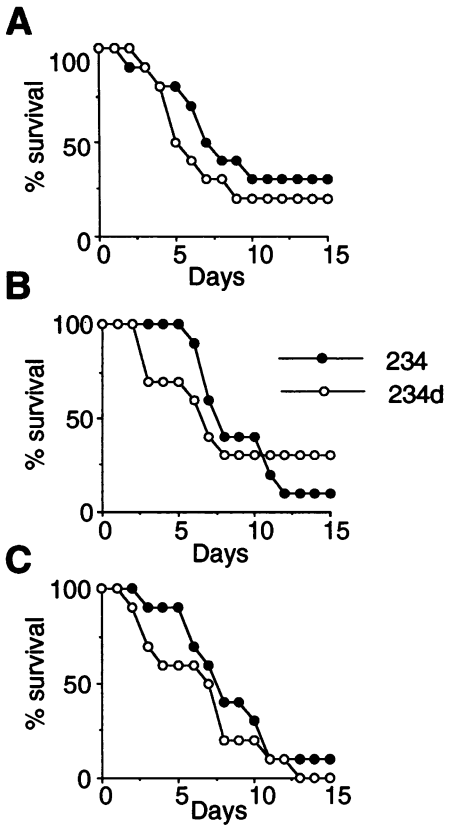

FIG. 3. Survival curves of mice inoculated by the intranasal route with strains 234 and 234d. Ten mice were infected with each strain in each of three experiments (A, B, and C).

isolates recovered from the lungs of mice inoculated with 237d were all elastase nonproducers by the elastin plate assay. Histological examination of lung tissue failed to show any differences between mice inoculated with strains 237 and 237d. Representative sections are shown in Fig. 4. Infection was widely distributed throughout the lung fields, involving many alveolar spaces. Although widespread, the individual colonies were small. A polymorphonuclear (PMN) leukocyte infiltrate was present around the colonies. Limited elastin degradation was observed in the pneumonic foci caused by infection with both the wild-type and AFAlp disruptant strains.

(ii) Intranasal model. Since animals were immunosuppressed for a longer period in the intranasal-inoculation model, it was necessary to add antibiotics to the drinking water and provide animals with sterile bedding and food. This ensured that mice given only corticosteroids remained healthy. Animals that were inoculated with strain 234 in the absence of immunosuppression showed no effect from exposure to conidia. The lowest level of inoculum of strain 234 given to immunosuppressed mice which gave reproducible survival curves was 8×10^5 conidia; only 30 to 50% of mice receiving 4×10^5 conidia developed respiratory illness (data not shown). There was no significant difference in the survival of mice inoculated with each of the fungal strains when experiments were analyzed both individually and together (Fig. 3). An attempt was made to assess the fungal load in mice by measuring CFU per gram of lung from infected animals, but there was wide variation in the counts from animals inoculated with the same strain which developed IPA at the same time after inoculation. In agreement with previous work on *A. fumigatus* (22), we conclude that measuring CFU is not a reliable indicator of fungal load in lung tissue.

No differences were observed in the histology for animals inoculated by the intranasal route with strain 234 or 234d (Fig. 4). Discrete fungal colonies were seen in large airways, filling the lumen, invading through the wall, and in some cases invading the adjacent branch of the pulmonary artery. The colonies were surrounded by a dense PMN leukocyte infiltrate in which a large number of cells appeared degenerate. Focal breakdown of elastin was seen in some bronchial and vessel walls at the site of fungal invasion (Fig. 4). Adjacent sections that were stained with hematoxylin and eosin showed that PMN leukocytes were often present at the sites of elastin breakdown.

To investigate the possibility that integration of the disruption vector results in an alteration in virulence, animals were inoculated with strains 237e and 234e, which are single-copy ectopic transformants, by the inhalation and intranasal routes, respectively. No difference in survival curves was observed when these groups were compared with animals inoculated with wild-type isolates (data not shown).

DISCUSSION

Previous attempts to define the pathogenicity determinants of aetiological agents of systemic mycoses, which include *Aspergillus* spp., *Candida* spp., and *Cryptococcus neoformans*, have been based on comparisons of wild-type strains with naturally occurring variants (11) or with mutants generated either by UV light or chemicals (13, 15, 21) and the revertants of some of these mutants (13). An inherent problem with these approaches is that such strains may carry additional mutations at other loci which could influence the infection process. In an effort to establish the relationship between virulence and the mating type of *C. neoformans*, Kwon-Chung et al. (12) used a series of backcrosses to construct near-isogenic strains which differed at the mating-type locus. However, as those authors acknowledged, this approach cannot prove a causal relationship between virulence and mating type because of the possibility of close linkage between a virulence-enhancing gene and the mating-type system. In this study, we compared clinical isolates of *A. fumigatus* with isogenic strains constructed by targeted disruption of a putative pathogenicity gene (24). This strategy should provide a rigorous test for determining the role of a gene in pathogenesis and has been used recently to refute the hypothesis that cutinase secreted by *Fusarium solani* var. *pisi*, a fungal phytopathogen, is required for disease development (23).

Disruption of the AFAlp gene was performed in two genetic backgrounds, as the relative contribution of virulence determinants may be strain dependent. The clinical isolates of *A. fumigatus* chosen for this work, 234 and 237, are both elastase producers and were obtained from geographically distant sites. Most isolates of *A. fumigatus* are morphologically indistinguishable but can be differentiated by RAPD analysis (1). We used RAPD analysis in this work for several reasons: because of the continual risk of airborne contamination, to ensure that the correct pairs of strains were being compared in each experiment, and to establish the identities of isolates recovered from the lungs of infected animals. The AFAlp gene disruption in strains 237d and 234d

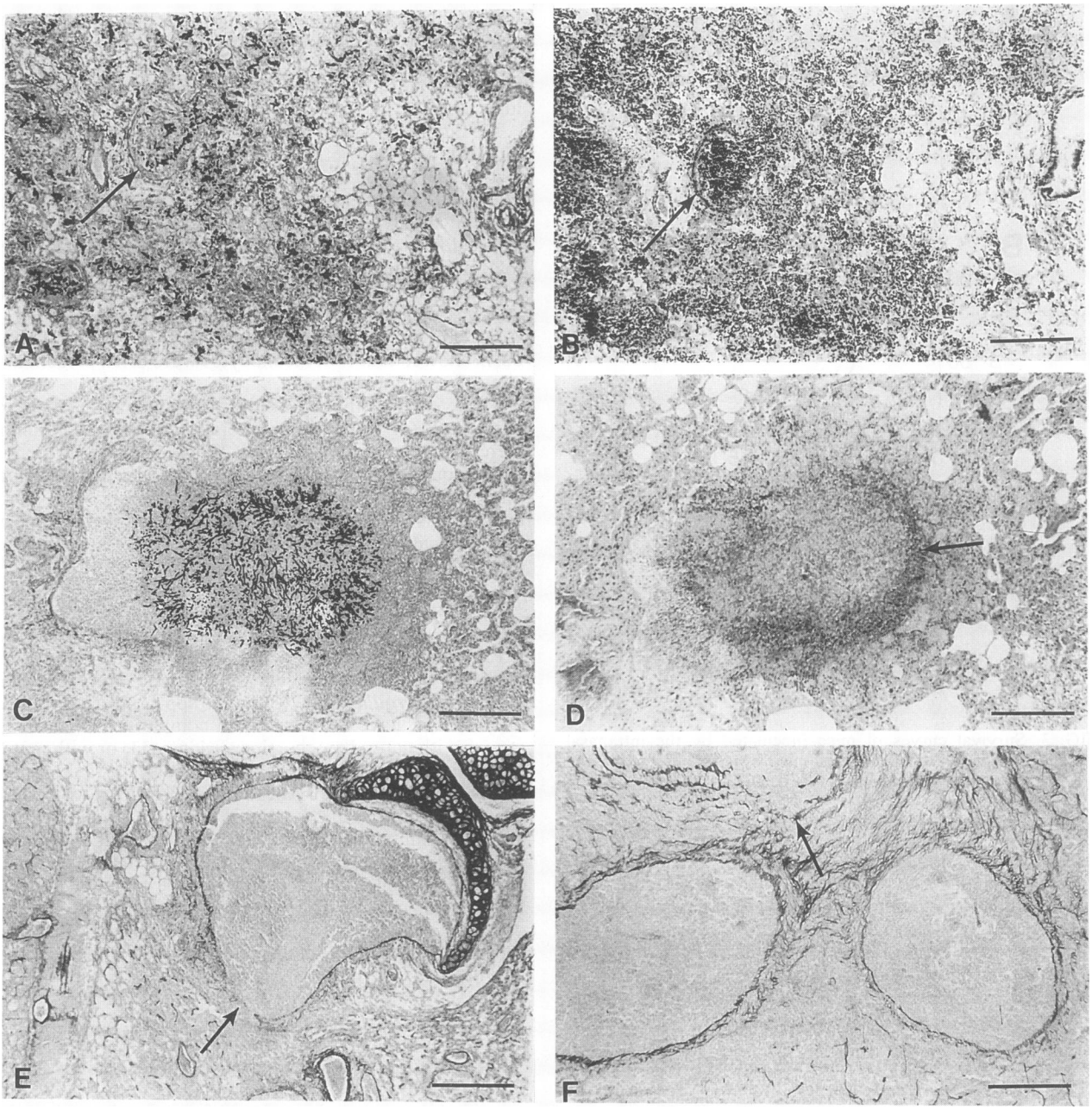

FIG. 4. (A and B) Serial paraffin sections of lung from a mouse inoculated by inhalation of airborne conidia. (A) Grocott's silver impregnation; (B) hematoxylin and eosin. The bronchiole (arrow) contains fungi (stained black in panel A) which are also dispersed through the parenchyma. An inflammatory infitrate consisting of PMN leukocytes (stained black in panel B) is present in affected areas. (C and D) As for panels A and B, respectively, but from a mouse inoculated by the intranasal route. Fungi and inflammatory cells (arrow) are centered on the airway but are not disseminated in the parenchyma. (E and F) Paraffin sections of mouse lung stained for elastic fibers. Animals were inoculated by airborne conidia (E) or nasal droplet (F). Focal disappearance of the elastic laminae (arrows) is visible in both preparations. All tissue sections shown are from animals inoculated with wild-type strains. Comparable results were obtained from animals inoculated with AFAlp disruptant strains. Bars = 100 μm.

results in the close proximity of two regions of identical sequence, a 504-bp *Kpn*I-*Sal*I fragment (Fig. 1). It is possible that these regions could undergo recombination leading to loss of the disruption vector and reconstitution of a functional AFAlp gene. All isolates recovered from animals inoculated with 234d and 237d were elastase nonproducers by the plate assay and AFAlp disruptants by the PCR assay. This demonstrates both that mice were not contaminated to any significant degree by environmental elastase-producing strains of *A. fumigatus* and that reversion to AFAlp production did not occur in the lungs of infected mice. Therefore, over the course of these experiments which involved multiple cycles of cell division during growth of the fungus in the host, the AFAlp disruptants were stable.

Initially, we attempted to replicate the model described by Kothary et al. (11), because they used it to demonstrate a

difference in both survival and tissue invasion between elastase-producing and nonproducing isolates. The major advantage of this model is that animals are inoculated by the inhalation of airborne conidia, thereby simulating the delivery of inoculum in the clinical setting. In our experiments, animals required more immunosuppression to induce IPA than was described by Kothary et al. (11). This difference may have been due to the different fungal and animal strains used in the studies. In contrast to Kothary et al. (11), who found that the majority of conidia of elastase nonproducing isolates failed to germinate in infected mice, most conidia of strain 237d did germinate within the lung (Fig. 4). The differences they observed between elastase-producing and nonproducing isolates were presumably due to mutations unconnected with the AFAlp gene. We thought that our failure to demonstrate a difference between strains 237 and 237d in terms of mortality or histology with this model might reflect the short interval between inoculation with the fungus and the onset of illness (Fig. 2). Therefore, we compared strains 234 and 234d by using a murine model of IPA in which the duration of illness is more prolonged. The model employed is a modification of that described by Dixon et al. (7); approximately 50% mortality occurs by day 7, and 70 to 80% mortality occurs by day 12. The animals are inoculated via the respiratory tract and immunosuppressed with corticosteroids. The drawback of this model is that the inoculum is administered as a suspension in saline, which does not replicate the clinical mode of infection. So as not to produce an overwhelming infection, we lowered the fungal inoculum and dose of immunosuppression to the limits that gave reproducible survival curves but were still unable to demonstrate a difference between the two strains.

An obvious explanation for the striking difference in survival curves between the two models is apparent from the lung tissue histology. Multiple small foci of infection were found throughout the lung parenchyma following the inhalation of airborne conidia, while in contrast, the pattern of disease was largely bronchocentric after the aspiration of a droplet containing conidia (Fig. 4). This suggests that while a suspension of conidia inhaled by mice tends to lodge in the major airways, individual airborne conidia, because of their small size (2 to 3 μm in diameter), reach the distal part of the airways, where they germinate and cause a fulminant fungal pneumonia.

All animals that became ill had evidence of respiratory distress (i.e., labored breathing and subcostal recession). Since this was observed only in mice that were immunosuppressed and inoculated with viable conidia, we conclude that respiratory disease was due to fungal growth and was not the result of other infections, mechanical effects of the inoculum, or allergic reactions to conidia. This conclusion is supported by the histological data obtained from animals in the experimental groups which demonstrated invasive fungal disease.

An important aspect of this work was the demonstration of elastinolysis in lung tissue from animals infected with the AFAlp disruptants. There are several potential causes of the degradation of elastin in lung tissue from animals inoculated with the AFAlp disruptants. First, growth conditions in the microenvironment of an *A. fumigatus* colony in the lung are unknown, so it is possible that the extremely alkaline or acid proteases reported by Reichard et al. (19) or other as-yet-uncharacterized proteases are responsible for the elastinolysis. Second, elastin breakdown may be caused by host enzymes such as PMN leukocyte elastase. Although PMN leukocytes were not always detected at the site of elastinolysis, this does not preclude the possibility that they were responsible for elastin degradation, since they may have migrated or autolyzed after the breakdown of elastin had occurred. Finally, the loss of elastin could be due to nonspecific chemical degradation.

Our results show that AFAlp does not contribute significantly to fungal virulence in these murine models of IPA. However, it is conceivable that AFAlp is one of a number of factors, each with a minor effect, that combine to facilitate disease progression (5). In addition, this study does not address the question of the role of AFAlp during infection of organs other than the lung (16). Moreover, the clinical relevance of our findings is uncertain. There are inherent limitations with both animal models: large doses of both fungus and immunosuppression are necessary to induce disease, and animals are immunosuppressed by corticosteroids, while clinically, the major risk factor for the acquisition of IPA is prolonged neutropenia (9). Accordingly, we intend to assess the pathogenicity of the AFAlp disruptants in neutropenic mice, which may require a lower level of fungal inoculum to produce disease.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Since submission of this paper, Monod et al. (M. Monod, S. Paris, J. Sarfati, K. Jaton-Ogay, P. Ave, and J.-P. Latgé, FEMS Microbiol. Lett. **106**:39–46, 1993) have reported that an *A. fumigatus* AFAlp disruptant retained virulence in cortisone-treated mice.

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