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Virulence of *Aspergillus fumigatus* Double Mutants Lacking Restrictocin and an Alkaline Protease in a Low-Dose Model of Invasive Pulmonary Aspergillosis

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To investigate the pathogenicity of *Aspergillus fumigatus* mutants lacking putative virulence factors, we have developed a new murine model of invasive pulmonary aspergillosis based on neutropenia, the major factor predisposing patients to this infection. Mice were treated with cyclophosphamide and inoculated by the intranasal route with 5×10^3 conidia, a significant reduction from inoculum levels used in previous models. Evidence for the production of the extracellular alkaline protease (Alp) in lung tissue was obtained by using a fungal transformant harboring an *alp*::*lacZ* reporter gene fusion. The pathogenicities of single mutant strains lacking either Alp or the ribotoxin restrictocin and of a double mutant strain lacking both proteins were assessed in this infection model. There were no significant differences between the mutant and the wild-type strains in terms of mortality or histological features. Inoculations with mixtures of conidia showed that the double mutant strain is slightly less virulent than the wild-type strain. We conclude that Alp and restrictocin are not important virulence determinants in pulmonary infection.

A number of animal models of aspergillosis have been developed for the evaluation of antifungal agents and the analysis of fungal virulence factors. These models vary with respect to the immunosuppressive regimens used and the means of administration of inoculum. Animals immunosuppressed with steroids (7, 9), cyclophosphamide (7), or gamma irradiation (12) have been inoculated by intravenous (23, 25) or intratracheal (28, 37) injection and by the inhalation of dry spores (13) or spore suspensions (36). In previous studies, we used two murine models of invasive pulmonary aspergillosis (IPA) to compare the pathogenicities of wild-type and mutant strains of *Aspergillus fumigatus* (26, 32). In one model, mice were inoculated by the inhalation of airborne conidia; in a second model, conidia were delivered intranasally as a suspension in saline. In both cases, corticosteroids were given on more than one occasion to impair both macrophage and neutrophil functions (22, 25). *A. fumigatus* mutants lacking either an extracellular elastase (the alkaline protease [Alp]) or the cytotoxin restrictocin remained pathogenic (26, 32), demonstrating that they are not important virulence determinants in these models.

One of the limitations of both models is that the large dose of fungal inoculum required to establish reproducible mortality does not reflect the numbers of conidia inhaled by patients. Given that there are 1 to 15 CFU of pathogenic *Aspergillus* spp. m^{-3} in the air (21) and the average pulmonary ventilation per day is 1.93 m^3 (10), the average human inhales approximately 30 spores per day. Increases in the levels of airborne *Aspergillus* conidia during construction work, which have often been noted prior to outbreaks of IPA, may raise this level 10-fold (29). Even so, these numbers are several orders of magnitude lower than doses used to inoculate mice (7, 18, 32). The infections produced by such large inocula could mask minor effects of virulence factors on fungal growth. Furthermore, in our previous work, mice were immunosuppressed with corticosteroids, whereas clinically, the major risk factor for IPA is profound and prolonged neutropenia. There is, therefore, a need for animal models of IPA which reflect the clinical condition more closely, and before excluding Alp and restrictocin as virulence factors in human disease, it was important to test the pathogenicity of mutants lacking these proteins in neutropenic mice. In steroid-treated mice, each fungal colony invading the lung tissue is surrounded by a dense neutrophil infiltrate (32). By reducing or eliminating this host response, we thought that it might be possible to reduce the number of conidia required to cause sufficient lung colonization to result in IPA. The importance of testing our strains in neutropenic animals was further emphasized by a report by Kolattukudy et al. (12) in which an Alp-deficient mutant of *A. fumigatus* was shown to be less virulent than a wild-type strain in neutropenic mice.

For restrictocin and Alp to act as virulence factors, these proteins must be produced during invasion of the lung. Restrictocin is the major antigen in the urine of patients with IPA (14, 15), and the *res* gene is expressed when the fungus is grown in a variety of culture media (27), indicating that this protein is produced constitutively. In contrast, production of Alp is subject to nitrogen, sulfur, and carbon catabolite repression as well as pH regulation (3, 31). Previous studies have used polyclonal anti-Alp antibodies to detect the enzyme in lung sections from a patient with IPA (20) and in germinating conidia and hyphae in the lungs of mice (12). However, it is conceivable that the staining detected by these approaches could have resulted from nonspecific antibodies to other fungal antigens produced in laboratory animals after the preimmune serum had been collected.

In this study, we developed a model of IPA in which neutropenia is maintained by repeated cyclophosphamide injections and the level of inoculum (administered by intranasal instillation) is lowered significantly from the levels used in

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previous work. We describe the use of an *alp*::*lacZ* transformant to provide strong evidence that Alp is produced during infection of the lung, the construction of a double mutant strain of *A. fumigatus* lacking both Alp and restrictocin, and the virulence of this and single mutants.

MATERIALS AND METHODS

Fungal strains and DNA extractions. *A. fumigatus* 237 was cultured from an open lung biopsy from a patient with IPA at Hope Hospital, Manchester, United Kingdom. Strains 342 and 363 were derived from strain 237 by single gene disruptions of the *alp* gene (31) and the restrictocin gene (26), respectively. DNA was isolated from *A. fumigatus* by a rapid extraction procedure (31).

Induction of neutropenia. White male CD1 mice (Charles River Breeders, Kent, United Kingdom), weighing 23 to 28 g, were immunosuppressed with cyclophosphamide (Farmitalia Carlo Erba Ltd., St. Albans, United Kingdom) at a dose of 150 mg kg of body weight^{-1} injected intraperitoneally on days −3, −1, 3, 6, and 9 and with one subcutaneous injection of hydrocortisone acetate (Boots, Nottingham, United Kingdom) at a dose of 112.5 mg kg^{-1} on day −1. In a second series of experiments, mice were immunosuppressed with cyclophosphamide (150 mg kg^{-1}) given intraperitoneally on days −3, 0, 3, and 6. Hydrocortisone acetate was administered as described above.

The animals were kept in sterile cages with filter tops and received sterile food and bedding. Tetracycline (1 mg ml^{-1}; Sigma Laboratories Ltd., Poole, United Kingdom) was added to the drinking water, which was changed twice daily. Leukocyte and differential counts from peripheral blood were monitored at intervals (days −3, 0, 3, 6, 10, and 13) to measure the degree of neutropenia. At each time point, blood was drawn from the tail veins of three mice which had been inoculated with saline only. Leukocyte counts were measured with a counter (model F1; Coulter Electronics Ltd., Luton, United Kingdom). Differential counts were determined from duplicate samples by using Giemsa-Wright-stained blood films.

Preparation of inocula. Each fungal strain was grown for 3 days at 37°C on Sabouraud dextrose medium in a 100-ml bottle. Conidia were harvested as a suspension in 10 ml of 0.01% Tween 80 and diluted 1/10 in 0.01% Tween 80 in saline. A 1-ml aliquot was centrifuged at 4,000 × *g*, and the conidia were suspended in 1 ml of 0.01% Tween 80 in saline. The concentration of spores in each sample was determined with a hemocytometer, and appropriate dilutions were made in the same solution.

Infection model. On day 0, mice were anesthetized by inhalation of diethyl ether and inoculated by the intranasal route with a suspension of *A. fumigatus* conidia (wild-type strain 237) in 30 μl of 0.01% Tween 80 in saline. Groups of 8 to 10 mice were inoculated with either 5×10^4, 1×10^4, 5×10^3, 2.5×10^3, 1×10^3, or 5×10^2 spores. In the first immunosuppressive regimen (cyclophosphamide on days −3, −1, 3, 6, and 9), inoculation with each level of inoculum was repeated on at least two separate occasions, except doses of 1×10^3 and 5×10^2 conidia, which were inoculated in only one experiment. Mice immunosuppressed on days −3, 0, 3, and 6 were inoculated with 5×10^3, 1×10^3, 5×10^2, and 2.5×10^2 spores. Inoculation with each level of inoculum was repeated once, except the lowest dose. Concurrent control groups consisted of mice that were immunosuppressed and inoculated with 30 μl of 0.01% Tween 80 in saline. The animals were observed twice each day for 15 days after inoculation, and mortality was recorded. Carbon dioxide was used to induce narcosis in mice with severe respiratory distress.

Histopathology. For histopathological examination, the right lung from each mouse was dissected and fixed in 10% (vol/vol) formaldehyde in physiological saline. Sections were stained with Grocott's methenamine silver nitrate and with hematoxylin and eosin by standard techniques.

Construction of a reporter gene. Primer alp14 (5′-GTAG GATCCGTGGTTTGAATAGA-3′) and the M13 reverse sequencing primer (5′-GTAAAACGACGGCCAGT-3′) were used to amplify a 490-bp fragment consisting of the *alp* promoter and the first seven codons of the *alp* gene from plasmid pID23. The 1.6-kb insert of pID23 contains *alp* coding region sequences and promoter sequences and was constructed from plasmid pID22 (30) by digestion with *Eco*RI and self ligation. Primer alp14 was designed to produce an in-frame fusion when ligated to the *Escherichia coli lacZ* gene and included a *Bam*HI restriction site at the 5′ end to facilitate cloning of the PCR product. The *Eco*RI site also used for the subsequent ligation was located in the polylinker present in pID23 and within the 490-bp product amplified from this plasmid. PCRs were carried out in 100-μl volumes containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-Cl (pH 8.0), 25 μM each dATP, dCTP, dTTP, and dGTP (Pharmacia Biotech Ltd., Milton Keynes, United Kingdom), 100 pmol of both primers, 2.5 U of Amplitaq (Perkin-Elmer Cetus, Norwalk, Conn.), and 20 ng of target DNA. Thermal cycling conditions consisted of 32 cycles of the following: 94°C for 30 s, 54°C for 45 s, and 72°C for 2 min. The 490-bp amplified product was digested with *Bam*HI and *Eco*RI and ligated into pUC18 to generate pID40. A 3.1-kb *Bam*HI fragment containing the *E. coli lacZ* gene (2, 35) was ligated in the correct orientation into the single *Bam*HI site of pID40 to create plasmid pID41. Nucleotide sequence analysis of this plasmid using the dideoxy chain termination method (24) and Sequenase 2 (United States Biochemical, Cleveland, Ohio) confirmed the identity of the cloned PCR product and that the fusion between codon 7 of *alp* and codon 8 of *E. coli lacZ* was in frame. The 2.4-kb *Sal*I fragment of pFOLT4R4 (19), which contains the *E. coli* hygromycin B (hyg B) phosphotransferase gene (*hph*) flanked by the *Aspergillus nidulans trpC* promoter and terminator sequences (5), was ligated into the single *Sal*I site of pID41 to generate pID42.

The plasmid pID42 containing the *alp*::*lacZ* translational fusion gene was used to transform *A. fumigatus* 237; previously described methods (34) with the following modifications were used. DNA (2.5 μg) was mixed with 3×10^7 protoplasts, and aliquots of the protoplast-DNA mix were plated in 5 ml of regeneration medium (*Aspergillus* minimal medium [4] containing 1.0 M sucrose, 1% glucose, and 5 mM ammonium tartrate) onto 15 ml of the same medium and then incubated at room temperature. Plates were overlaid after 16 h with 10 ml of regeneration medium containing 40 μl of a 200-mg ml^{-1} concentration of hyg B to give a final concentration of 267 μg ml^{-1}. Transformants were visible after 24 to 48 h of incubation at 37°C. DNA was extracted from each transformant, digested with *Xho*I, and analyzed by Southern hybridization to identify transformants in which the reporter gene had integrated at the *alp* locus.

To assess the activity of the *alp*::*lacZ* fusion in *A. fumigatus*, approximately 2×10^7 conidia of transformant 747 were used to inoculate 100 ml of 1% yeast carbon base (Difco Laboratories Ltd., East Molesey, United Kingdom), 1% glucose, 0.2% elastin (Fluka Chemicals Ltd., Gillingham, United Kingdom), and 50 mM Na borate (pH 7.6). After growth at 37°C for 3

days, mycelial cultures were prepared and assayed for β-galactosidase (β-gal) activity as described previously (35).

Lung infection with an *alp*::*lacZ* transformant. Groups of six mice were inoculated with 5×10^3 conidia of strain 747 or 237. To assess the transcriptional activity of the *alp* promoter at different stages of infection, two mice from each group were sacrificed on days +3, +7, and +10. The lungs were fixed in 10% (vol/vol) formaldehyde in physiological saline, and the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 0.5 μg ml^{-1}) was added to sections. Neutral red was added as a counterstain to visualize nuclei in the host tissue. Serial sections were stained with Grocott's methenamine silver nitrate by standard techniques.

Construction of a double mutant. The plasmid pID155 was constructed for the replacement of the *alp* gene in a *res* mutant strain. A 2.3-kb DNA fragment containing 1.3 kb of the TR1 promoter sequences from *Trichoderma reesei* (1) and 0.55 kb of the *trpC* terminator sequences from *A. nidulans* flanking the *Streptoalloteichus hindustanus ble* (8) gene was amplified from plasmid pUT737 (1) by using oligonucleotide primers pUT1 (5′-ATGGATCCGGTACCAAGGGTCAACATG-3′) and pUT2 (5′-ATGGATCCGGTACCTACGTGAATG-3′). Both primers contained *Kpn*I restriction sites at the 5′ termini to facilitate cloning of the PCR product. PCRs were carried out in 100-μl volumes as described above. Thermal cycling conditions consisted of 32 cycles of the following: 94°C for 30 s, 54°C for 45 s, and 72°C for 2 min. The 2.3-kb amplified product was gel purified and digested with *Kpn*I.

Plasmid pID23, which contains a 1.6-kb fragment encoding most of the *alp* gene, was digested with *Kpn*I to remove an internal 242-bp fragment. This fragment encodes a region containing two of the three residues which form the catalytic site of the Alp protein (aspartic acid residue 31 and histidine residue 72) (11). The 242-bp fragment was replaced with the 2.3-kb *Kpn*I-digested PCR product to create plasmid pID155. Digestion of pID155 with *Eco*RI and *Pst*I generated a 3.8-kb linear fragment containing the phleomycin cassette flanked by 858 bp of *A. fumigatus* sequences on the 5′ side and 655 bp on the 3′ side. The resulting linear DNA fragment was purified by gel electrophoresis and used to transform *A. fumigatus* 363, as described above, except that plates were overlaid with 10 ml of regeneration medium containing 60 μl of a 50-mg ml^{-1} concentration of phleomycin (Cayla, Toulouse, France) to give a final concentration of 100 μg ml^{-1}. DNA was extracted from transformants, digested with *Xho*I, and analyzed by Southern hybridization.

Pathogenicity of mutants. The pathogenicities of the *alp* disruptant strain 342 and the *res* disruptant strain 363 were assessed in neutropenic mice in three separate experiments. In two experiments, groups of 8 to 10 mice were inoculated with 5×10^3 conidia of either the wild type or disruptant strains. In a further experiment, eight mice were inoculated with 2.5×10^3 conidia of either strain. The pathogenicity of the *alp res* double mutant strain (417) was tested in two experiments in which groups of eight mice were inoculated with 5×10^3 spores (in 30 μl of 0.01% Tween 80 in saline) of wild-type strain 237 or strain 417. Mice were observed for 15 days, mortality was recorded, and the lungs of mice inoculated with disruptant strains were dissected. The right lung from each animal was used for histological examination.

Infection with mixed inocula. Equal volumes of suspensions containing 5×10^3 conidia per 30 μl were mixed to prepare inocula consisting of equal numbers of strain 237 and strain 342 or 363. The inocula were diluted 1:100 in saline, plated onto Sabouraud dextrose medium plates containing 50 mg of chloramphenicol $liter^{-1}$ (S/C plates), and incubated at 37°C for 48 h. A minimum of 100 colonies from each mixed inoculum were replated onto *Aspergillus* minimal medium (4) containing 1% glucose, 5 mM ammonium tartrate, 1% agar, and 200 μg of hyg B ml^{-1} to check the ratio of hyg B-sensitive (wild-type) spores to hyg B-resistant (disruptant) spores in the inocula. Groups of six mice were inoculated with 30 μl of each of the mixed inocula. Mortality was recorded, and the lungs of five mice in each group were dissected. Each lung was homogenized in 500 μl of saline and diluted 1:5 in saline. Aliquots (200 μl) were plated onto S/C plates and incubated at 37°C for 48 h. Colonies (100 per mouse) were single conidia purified and then tested for sensitivity to hyg B by replating onto media as described above. The ratio of sensitive to resistant colonies was calculated for each pair of lungs.

For mixed infections involving the *alp res* double mutant strain 417 (phleomycin and hyg B resistant), suspensions of 2×10^4 conidia per 30 μl were prepared for strains 237 and 417. The ratio of wild-type to mutant spores in the inoculum was checked as described above, except that colonies were replated onto media containing 100 μg of phleomycin ml^{-1} and onto media containing 200 μg of hyg B ml^{-1}. A group of six mice was inoculated with the mixed inoculum, and fungal colonies were recovered from the lungs and purified as described above. Single colonies (100 from each mouse) were tested for sensitivity to phleomycin and to hyg B.

Statistical analysis. The log rank method was used to analyze the survival of mice for statistical significance. For the mixed-inoculum experiments, mean values and standard deviations were determined and analyzed by using Student's *t* test.

RESULTS

Induction of neutropenia. Mice became neutropenic 3 days after the initial injection of cyclophosphamide. By day 0 (day of inoculation), the mean total leukocyte count had fallen to 0.44×10^9 cells $liter^{-1}$ (from 6.9×10^9 cells $liter^{-1}$ on day −3). Differential counts of blood smears showed a reduction of neutrophils to <30% of total leukocytes on day 0. Neutropenia was maintained for at least 10 days.

Experimental infection and histopathology. In the first series of experiments, cyclophosphamide was administered on days −3, −1, 3, 6, and 9. When groups of 8 to 10 immunosuppressed mice were inoculated with either 5×10^4, 1×10^4, or 5×10^3 conidia, >75% mortality was observed in replicate experiments. In contrast, inocula of 2.5×10^3, 1×10^3, and 5×10^2 conidia produced mortality that was not reproducible or resulted in <37.5% mortality. These sizes of inocula were not used in subsequent experiments. Mice were sacrificed when they developed all of the following symptoms of IPA: ruffled fur, immobility or hunched posture, respiratory distress, and severe subcostal recession. Control mice that were immunosuppressed and inoculated with saline did not develop any of these symptoms.

In a second series of experiments, cyclophosphamide was administered at 3-day intervals in an attempt to further reduce the level of inoculum. Groups of 8 to 10 mice were immunosuppressed with cyclophosphamide on days −3, 0, 3, and 6 and inoculated with 5×10^3, 1×10^3, 5×10^2, or 2.5×10^2 conidia. In mice inoculated with 5×10^3 and 1×10^3 conidia, mortalities of >50% were observed, while at the lower doses of inocula, the mortalities were variable. However, a persistent problem was the occasional death of immunosuppressed, noninoculated mice. Although such mice did not develop any symptoms of IPA, this regimen was not used in further experiments.

Cyclophosphamide immunosuppression on days −3, −1, 3,

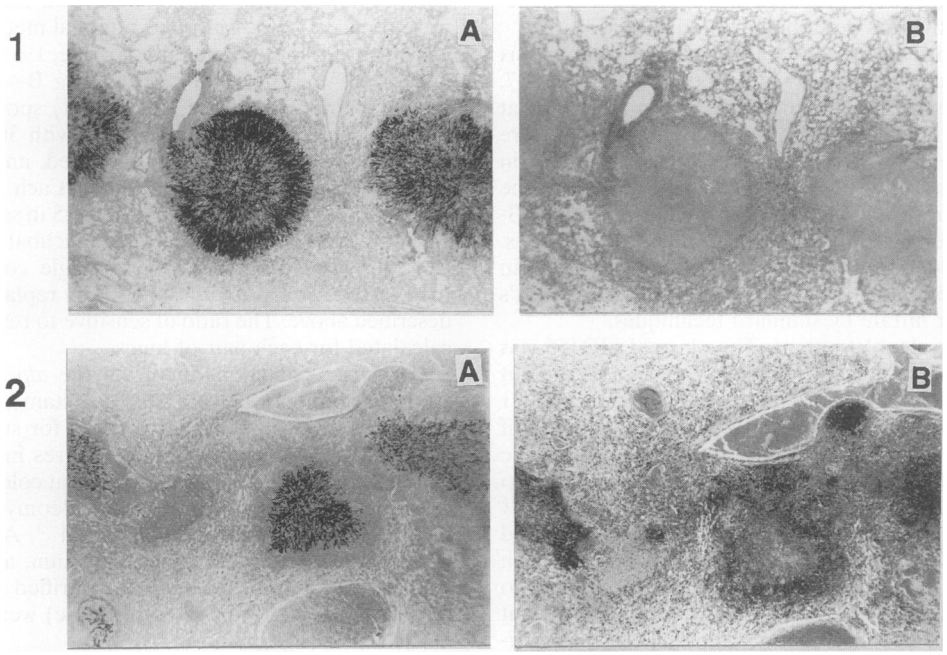

FIG. 1. Serial paraffin sections taken from the lungs of mice 7 days after inoculation with strain 237. Sections were stained with Grocott's methenamine silver nitrate (A) or with hematoxylin and eosin (B). Mice were immunosuppressed with a combination of cyclophosphamide and hydrocortisone (row 1) or with hydrocortisone alone (row 2). In sections from mice immunosuppressed with hydrocortisone alone, fungal colonies (stained black in panel 2A) are surrounded by an inflammatory infiltrate of neutrophils (stained black with hematoxylin and eosin in panel 2B). Magnification, ×57.5.

6, and 9 and an inoculum of 5×10^3 conidia was chosen for all subsequent work. In mice inoculated with strain 237, this level of inoculum resulted in >75% mortality by day 15, with most deaths occurring on days 4 to 7. (A typical survival curve is shown in Fig. 4.) Representative histological sections of lung tissue from mice inoculated with 5×10^3 conidia are shown in Fig. 1. Sections stained with Grocott's methenamine silver nitrate confirmed IPA by the presence of numerous dense fungal colonies within the lung tissue. These colonies varied in size, morphology, and density. In mice immunosuppressed by steroids alone, all fungal colonies were surrounded by a dense neutrophil infiltrate (32), whereas in this model, the neutrophil response was virtually absent (Fig. 1). To calculate the number of individual fungal colonies present in the lungs of mice inoculated with 5×10^3 conidia, sections were taken at 2-μm intervals across the entire lungs of each of three mice. The total volume of lung tissue colonized by fungal hyphae was determined. On the basis of the mean lung volume and the mean fungal colony volume, totals of 88, 72, and 11 colonies were estimated to be present in the three lungs, respectively.

Construction and characterization of the *alp*::*lacZ* transformant. Plasmid pID42 contains a selectable marker for hyg B resistance and the *alp*::*lacZ* translational fusion gene, consisting of 490 bp of the promoter of *alp* with the first seven codons of *alp* fused in frame to the *E. coli lacZ* gene (Fig. 2). pID42 was used to transform strain 237 to hyg B resistance. Southern analysis of 18 transformants, probing *Xho*I-digested genomic DNA with the 490-bp fragment of the *alp* promoter and the first seven codons of the *alp* gene, identified one transformant, strain 747, in which pID42 had integrated as a single copy at the *alp* locus (data not shown). This integration produced an *Xho*I fragment of 12.1 kb, which replaced the wild-type *Xho*I fragment of 3.5-kb (Fig. 2). The integration event placed the *alp*::*lacZ* fusion under the control of all of the chromosomal 5′ regulatory sequences, including any that lie upstream of the 490-bp cloned sequence.

To confirm that the expression of the *alp*::*lacZ* translational fusion was regulated in the same way as the *alp* gene, strains 237 and 747 were grown in liquid culture containing elastin, with or without 5 mM ammonium tartrate. The production of Alp is derepressed during growth on elastin and is repressed by ammonium (31). The media also contained 1% glucose, sufficient to repress the endogenous β-gal activity of *A. nidulans* (35). After growth at 37°C for 3 days, mycelial extracts were assayed for β-gal activity. The β-gal activity of transformant 747 was approximately 1,000-fold greater than that of the parental strain 237 when elastin was the sole nitrogen source but was below the limit of detection when ammonium was included in the growth medium (data not shown).

Inoculation with the *alp*::*lacZ* transformant. To analyze the transcriptional activity of the *alp* promoter during growth of *A. fumigatus* in the lung, two groups of six mice were inoculated with strain 747 or strain 237. Two animals from each group were sacrificed on days +3, +7, and +10, and sections of lung tissue stained for fungal hyphae (with Grocott's methenamine silver nitrate) and for β-gal activity (with X-Gal). In both groups and at each time point, branching septate hyphae were observed growing into and through lung tissue (Fig. 2). In sections from mice inoculated with strain 237, there was no detectable β-gal activity, while in mice inoculated with strain 747, β-gal activity was indicated by blue staining within the hyphae. At day +3, this staining was seen throughout the fungal colonies, whereas at days +7 and +10, staining was limited to the periphery of the colonies (Fig. 2).

Construction and analysis of the *alp res* double mutant. Plasmid pID155, in which a 242-bp internal region of the *alp*

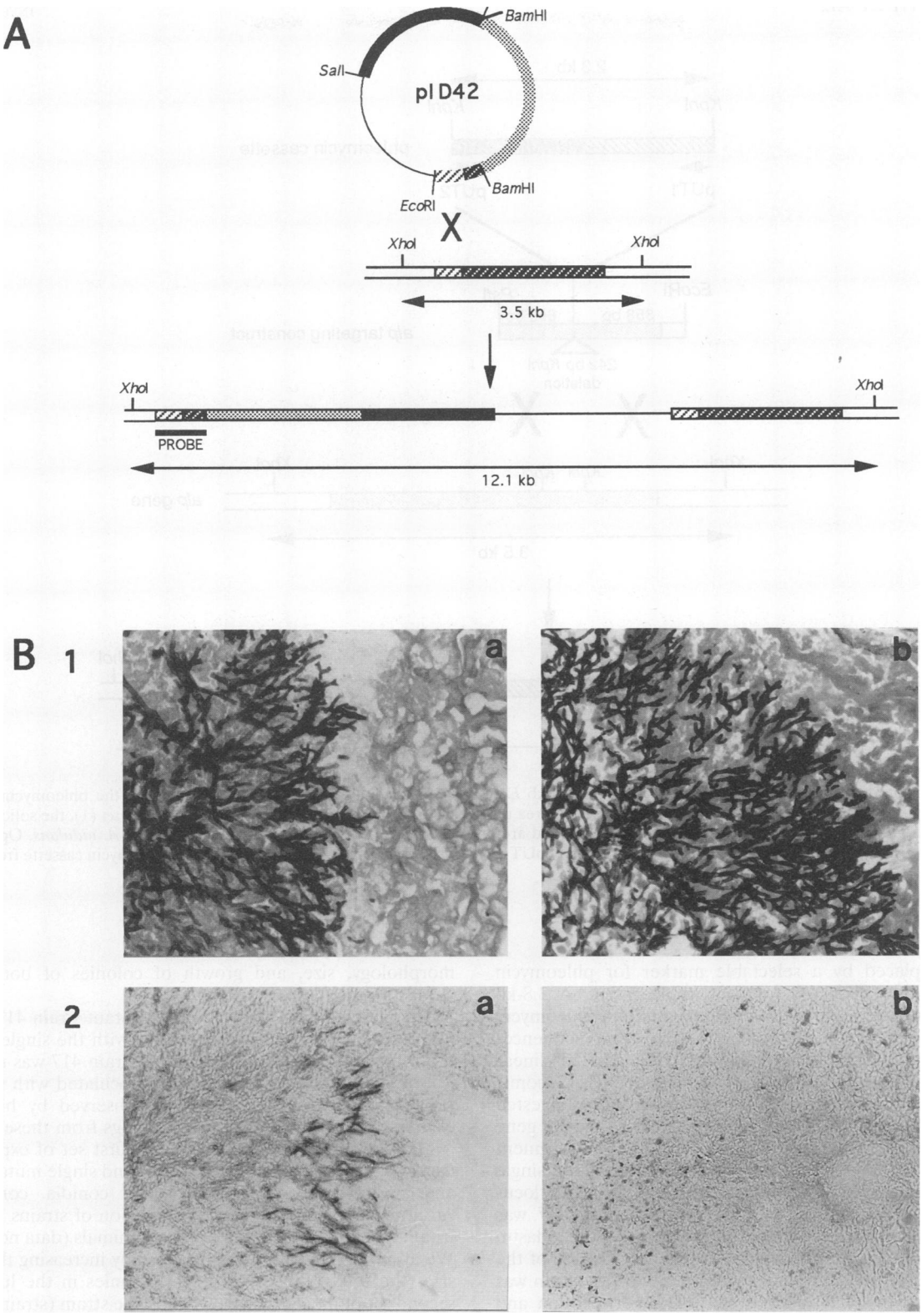

FIG. 2. (A) Targeting of the *alp*::*lacZ* fusion gene. The hatched area represents a 490-bp region of the *alp* promoter, the darkly hatched area represents the coding region of *alp*, the dotted area represents a 3.1-kb *Bam*HI fragment containing the *E. coli lacZ* gene, the thin line represents pUC18, and the solid black area represents the 2.4-kb *Sal*I fragment containing a selectable marker for resistance to hyg B (5). The horizontal lines with arrows show the expected sizes of *Xho*I restriction fragments before and after integration of pID42 at the *alp* locus. (B) Representative examples of lung tissue from neutropenic mice 7 days after inoculation with the *alp*::*lacZ* transformant strain 747 (a) or the wild-type strain 237 (b). Serial sections were stained with Grocott's methenamine silver nitrate to indicate the position of hyphae (panels in row 1) and incubated with the chromogenic substrate X-Gal, which is cleaved by β-galactosidase to liberate a blue dye (panels in row 2). Neutral red was employed as a counterstain to visualize nuclei. Magnification, ×230.

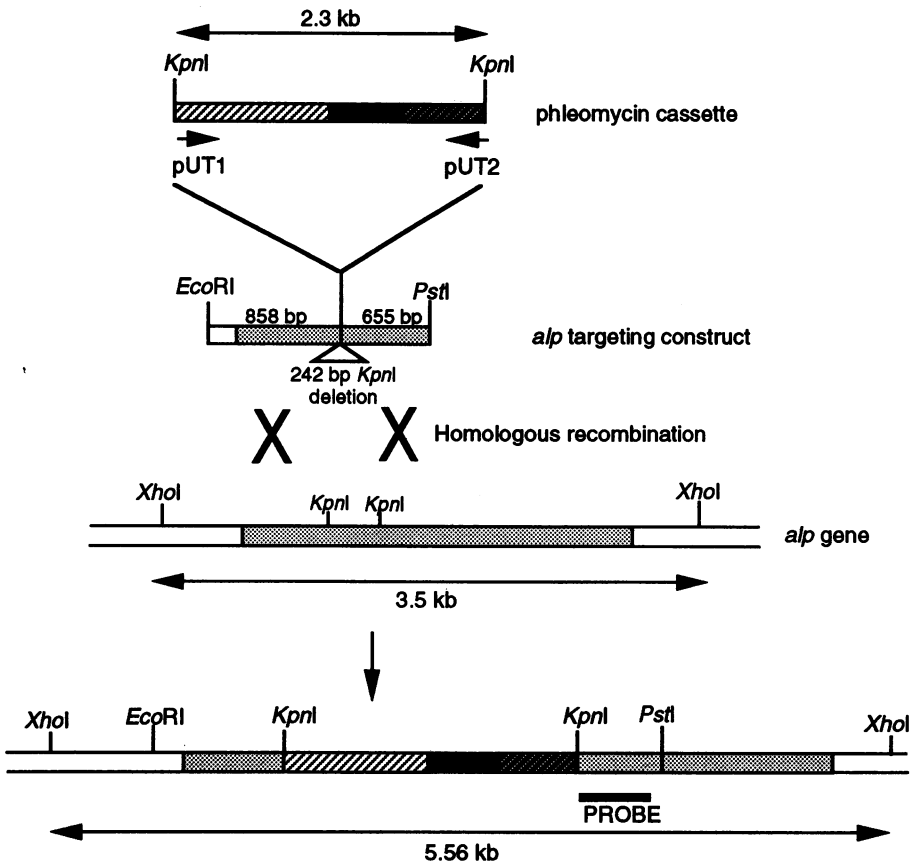

FIG. 3. Disruption of the *alp* gene by integration of a 3.8-kb *Eco*RI-*Pst*I fragment of plasmid pID55 containing the phleomycin resistance cassette. The spotted area represents the *alp* gene, the hatched area represents the TR1 promoter sequences from *T. reesei* (1), the solid black area represents the *S. hindustanus ble* gene (8), and the dark hatched area represents the *trpC* terminator sequences from *A. nidulans*. Open regions represent *A. fumigatus* sequences flanking the *alp* gene. Primers pUT1 and pUT2 were used to amplify the 2.3-kb phleomycin cassette from plasmid pUT737 (1).

gene was replaced by a selectable marker for phleomycin resistance, was constructed for gene replacement. A 3.8-kb *Eco*RI-*Pst*I fragment of this plasmid consists of a phleomycin resistance cassette flanked by 858 bp of *A. fumigatus* sequences at the 5′ end and 655 bp at the 3′ end (Fig. 3). This linear fragment was used to transform *A. fumigatus* 363. Genomic DNA of 28 phleomycin-resistant transformants was digested with *Xho*I and probed with a 570-bp fragment of the *alp* gene (data not shown). In 16 transformants, a 3.5-kb *Xho*I fragment was replaced with a 5.56-kb fragment, indicating that a single copy of the targeting construct had integrated at the *alp* locus (Fig. 3). One such transformant, referred to as strain 417, was chosen for further study. This strain failed to degrade elastin when grown on elastin medium, indicating the absence of the Alp protein (31). Disruption of the *res* gene in this strain was also confirmed by PCR analysis (26). The germination and radial growth rates of the double mutant were indistinguishable from those of the wild-type parent strain.

Virulence of mutants. The pathogenicities of strain 237 and the two single mutants, strains 342 (*alp*) and 363 (*res*), were compared in two separate experiments in which mice were inoculated with 5×10^3 spores. There was no significant difference in the survival of mice inoculated with each of the strains when analyzed within each experiment and as pooled data (Fig. 4). There were no obvious differences in the morphology, size, and growth of colonies of both mutant strains in lung tissue.

The pathogenicity of the double mutant strain 417 (*alp res*) was assessed in two experiments. As with the single mutants, the survival of mice inoculated with strain 417 was not significantly different from that of mice inoculated with strain 237 (Fig. 4), and no differences were observed by histological examination of infected regions of lungs from these mice.

Mixed-inoculum infections. In the first set of experiments, using conidium mixtures of wild-type and single mutant strains and an inoculum level of 5×10^3 conidia, considerable variation was observed in the proportion of strains recovered from infected lung tissue of different animals (data not shown). We attempted to lower this variation by increasing the sample size (the total number of fungal colonies in the lung). In a second set of inoculations, the wild-type strain (strain 237) was mixed with the double mutant strain (strain 417), and inocula of 2×10^4 conidia were used to infect six mice; of these, five were dissected and their lung homogenates were plated. One hundred colonies recovered from the lungs of each mouse were streaked onto S/C plates, and single colonies were then replated onto selective media to assess the relative proportions of wild-type and mutant strains in the lungs. There was a small decrease in the proportion of mutant colonies recovered from each animal, which was not significant at the 2% level (P =

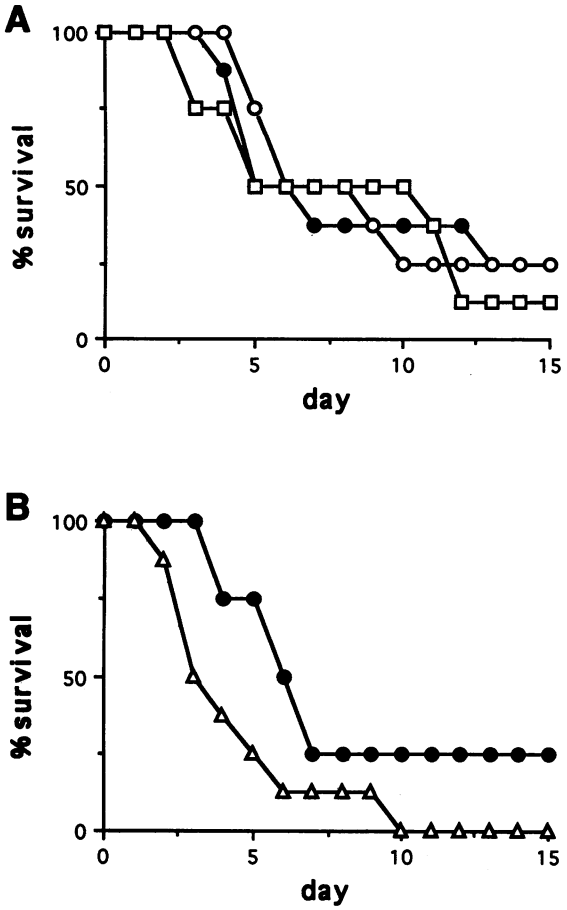

FIG. 4. Survival curves of mice inoculated with the wild-type strain 237 (closed circles), the *alp* mutant strain 342 (open squares), the *res* mutant strain 363 (open circles) or the *alp res* double mutant strain 417 (open triangles). Panels A and B represent two separate experiments in which eight mice were inoculated with each strain.

0.023). However, as P was less than 0.05, this decrease is significant if considered at the 5% level (Table 1).

DISCUSSION

In this study, we developed a model of IPA in which mice are rendered neutropenic by cyclophosphamide and the level of inoculum is reduced to 5×10^3 conidia. The aims of this model were twofold: to reproduce the most common form of immunosuppression that predisposes humans to IPA and to reduce the level of fungal inoculum required to produce infection.

In immunocompetent humans, two sequential lines of defense are known to act against *A. fumigatus*. Macrophages eradicate conidia in vivo, prevent germination, and kill conidia ex vivo, while neutrophils protect against the fungus by killing hyphae (6, 25, 36). It has been shown that neutropenia alone is insufficient to render mice susceptible to *A. fumigatus* unless the macrophage line of defense is overcome by high challenge doses, activated conidia, or cortisone suppression of macrophage conidiacidal activity (7, 25). Therefore, in our model of IPA, both cyclophosphamide and hydrocortisone acetate were administered to animals to suppress both lines of host defense against *A. fumigatus*. Cyclophosphamide eliminates neutrophils, while corticosteroids impair neutrophil function by reducing their mobilization (25) and suppressing the hyphal

TABLE 1. Proportion of strains in mixed inoculum and recovered from infected lung tissue

Strain	% Of colonies recovered from:		P value
	Inoculum[a]	Lungs[b]	
237	44.0	54.6 ± 8.22	0.023
417 (*alp res*)	56.0	45.4 ± 8.22	0.023

[a] Each mouse was inoculated with a mixture of 2×10^4 conidia of strains 237 and 417.

[b] Values are means ± standard deviations of results with five mice; 100 purified colonies per mouse were analyzed.

damage caused by the release of oxidative metabolites (22); patients receiving high doses of cortisone are also predisposed to IPA. By inhibiting both lines of defense against *A. fumigatus*, we were able to reduce significantly the number of fungal conidia required to produce infection from levels used in previous models of IPA (7, 32). Inoculation with 5×10^3 conidia consistently produced >75% mortality. By examining serial sections taken from entire lungs, an average of 57 colonies was estimated to be present in each lung, representing approximately 1/100 of the initial inoculum. If individual colonies arise from single conidia, presumably the remainder of the inoculum is exhaled or swallowed or fails to initiate a mycelium.

Previous attempts to demonstrate Alp production during host infection have been based on immunocytochemical techniques (12, 20, 30). There are inherent problems with these techniques because laboratory animals can develop nonspecific polyclonal antibodies to fungal antigens other than the injected Alp antigen after removal of the preimmune serum, resulting in nonspecific staining (30). To circumvent these problems, we used a β-gal staining system to assay the expression of an *alp::lacZ* fusion gene. β-Gal activity was detected throughout the colonies at early stages of infection but was limited to colony peripheries in sections of large colonies at later stages of infection, probably because cells at the edge of a colony are metabolically more active than those at the center.

Virulence tests with *A. fumigatus* mutants lacking restrictocin or Alp or both proteins showed that these extracellular products do not have major effects on fungal virulence in neutropenic mice. The same results were obtained for both single mutants in other models of IPA, which involved immunosuppression with corticosteroids (16, 17, 26, 32). Our results therefore contradict those of Kolattukudy et al. (12), who reported reduced virulence of an Alp-deficient mutant in neutropenic mice. However, their mutant was generated by chemical mutagenesis, and the reduction in pathogenicity they observed was probably due to an additional uncharacterized mutation(s).

Mixed-inoculum infections have revealed that the growth of *A. nidulans lysA* strains is significantly less than that of prototrophic strains in the lungs of neutropenic mice (33). This reduction was not reflected in a significant difference in the survival of mice inoculated with the auxotrophic mutant strain alone, which suggests that data from mixed infections might provide a more sensitive measure of virulence than mortality. The growth of the *A. fumigatus* wild-type strain and that of the *alp res* double mutant strain were compared by inoculating mice with a mixture of the two strains and characterizing isolates after recovery from symptomatic animals. An advantage of these experiments is that fewer animals are required to assess virulence. Potential drawbacks include the possibility that a wild-type strain might cross-feed an adjacent mutant

strain or that the isolation procedure favors the recovery of one of the strains. Given these provisos, the results indicate that the double mutant is slightly less virulent than the wild-type strain. It is not clear whether this is due to one mutation or the loss of both proteins, but the effect is minor, and further investigation with a view to exploiting the protein(s) as a target for antifungal drugs is probably not warranted.

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