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Gliotoxin Is a Virulence Factor of *Aspergillus fumigatus*: *gliP* Deletion Attenuates Virulence in Mice Immunosuppressed with Hydrocortisone▽

Janyce A. Sugui,[1]† Julian Pardo,[2]† Yun C. Chang,[1] Kol A. Zarember,[3] Glenn Nardone,[4] Eva M. Galvez,[5] Arno Müllbacher,[6] John I. Gallin,[3] Markus M. Simon,[2] and Kyung J. Kwon-Chung[1]*

Laboratory of Clinical Infectious Diseases,[1] Laboratory of Host Defense,[3] and Research Technology Branch,[4] National Institutes of Health, Bethesda, Maryland; Max-Planck-Institute for Immunology, Freiburg, Germany[2]; Institut für Physikalische Chemie, Freiburg Universität, Freiburg, Germany[5]; and John Curtin School of Medical Research, Australian National University, Canberra, Australia[6]

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Gliotoxin is an immunosuppressive mycotoxin long suspected to be a potential virulence factor of *Aspergillus fumigatus*. Recent studies using mutants lacking gliotoxin production, however, suggested that the mycotoxin is not important for pathogenesis of *A. fumigatus* in neutropenic mice resulting from treatment with cyclophosphomide and hydrocortisone. In this study, we report on the pathobiological role of gliotoxin in two different mouse strains, 129/Sv and BALB/c, that were immunosuppressed by hydrocortisone alone to avoid neutropenia. These strains of mice were infected using the isogenic set of a wild type strain (B-5233) and its mutant strain (*gliP*Δ) and the the *glip* reconstituted strain ($gliP_R$). The *gliP* gene encodes a nonribosomal peptide synthase that catalyzes the first step in gliotoxin biosynthesis. The *gliP*Δ strain was significantly less virulent than strain B-5233 or $gliP_R$ in both mouse models. In vitro assays with culture filtrates (CFs) of B-5233, *gliP*Δ, and $gliP_R$ strains showed the following: (i) deletion of *gliP* abrogated gliotoxin production, as determined by high-performance liquid chromatography analysis; (ii) unlike the CFs from strains B-5233 and $gliP_R$, *gliP*Δ CFs failed to induce proapoptotic processes in EL4 thymoma cells, as tested by Bak conformational change, mitochondrial-membrane potential disruption, superoxide production, caspase 3 activation, and phosphatidylserine translocation. Furthermore, superoxide production in human neutrophils was strongly inhibited by CFs from strain B-5233 and the $gliP_R$ strain, but not the *gliP*Δ strain. Our study confirms that gliotoxin is an important virulence determinant of *A. fumigatus* and that the type of immunosuppression regimen used is important to reveal the pathogenic potential of gliotoxin.

Invasive aspergillosis (IA) has become increasingly prevalent as the population of immunocompromised patients has increased (6, 15). Of about 20 species of *Aspergillus* reported to cause IA, *Aspergillus fumigatus* is the most frequently isolated species regardless of the underlying risk factors (6, 15). *A. fumigatus* produces a variety of secondary metabolites, including gliotoxin (13), and this mycotoxin has received considerable attention for over 2 decades as a putative virulence factor based on the following observations: (i) up to 93% of *A. fumigatus* strains recovered from cancer patients with IA produced gliotoxin (17) compared to less than 20% of the environmental isolates (8); (ii) *A. fumigatus* is the most prolific producer of gliotoxin among the pathogenic *Aspergillus* species tested (17); (iii) gliotoxin is immunosuppressive and proapoptotic for mammalian cells (9, 19–21, 30, 39); (iv) gliotoxin inhibits the NADPH oxidase activity responsible for the neutrophil oxidative burst (38); (v) circulating gliotoxin is readily detected in experimental aspergillosis and in sera from patients with IA, while it is only occasionally detected in patients without any evidence of IA (16); (vi) mice infected with a non-gliotoxin-producing strain survived longer than those infected with a genetically unrelated gliotoxin producer (36).

Although gliotoxin was identified in cultures of *A. fumigatus* during the 1940s (29) and was first synthesized in 1976 (11), it was only recently that its biosynthetic pathway was proposed (12). Using the bioinformatics approach, Gardiner and Howlett have recently identified a gene cluster in the *A. fumigatus* genome that is similar to the biosynthetic cluster of sirodesmin, a metabolite produced by the plant pathogen *Leptosphaeria maculans* (12). The first proposed step in the gliotoxin biosynthetic pathway is catalyzed by a nonribosomal peptide synthase encoded by the *gliP* gene. Two recent studies have shown that deletion of *gliP* in the *A. fumigatus* genome-sequencing strain, AF293, prevented the synthesis of gliotoxin but did not attenuate the virulence of the deletant strains (5, 14). A similar finding was reported with the deletion of *gliZ*, a transcriptional regulator in the gliotoxin biosynthetic pathway (3). In these studies, virulence was evaluated using neutropenic BALB/c and ICR mice that had been immunosuppressed with a combination of cyclophosphamide and cortisone acetate (3, 5, 14). An independent study has shown that the combination of these two drugs causes the immunosuppression to be so severe that even the uninfected control mice exhibited in-

* Corresponding author. Mailing address: Molecular Microbiology Section, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892. Phone: (301) 496-1602. Fax: (301) 480-3240. E-mail: june_kwon-chung@nih.gov.

† J.A.S. and J.P. contributed equally to this study.

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creased morbidity (28). Considering the inhibitory effect of gliotoxin on the oxidative burst of neutrophils, a neutropenic-mouse model may not be suitable for detecting the difference in virulence between the wild type and the gliotoxin-lacking mutant strains.

Previously, we have shown that Bak, a member of the pro-apoptotic Bcl-2 family, is the primary intracellular target in gliotoxin-induced apoptosis in vitro and that Bak knockout mice, immunosuppressed with corticosteroid, are more resistant to the *A. fumigatus* strain B-5233 than wild-type mice (26). It was thus our aim to investigate the significance of gliotoxin in the pathobiology of *A. fumigatus* by deleting the *gliP* gene in the strain B-5233, a highly virulent clinical isolate (37) able to grow on defined minimal medium, unlike strain AF293, which requires vitamin complex for normal growth (33a). The results presented here provide evidence that deletion of *gliP* in strain B-5233 results in a mutant unable to synthesize gliotoxin, as is the case with strain AF293, and that shows a reduced ability to damage target cells, fails to inhibit the oxidative burst in neutrophils, and is significantly less virulent for mice than the wild-type strain. Furthermore, our study stresses the importance of the type of immunosuppressive regimen used for evaluating the role of fungal toxins in pathogenesis and that the degree of susceptibility to aspergillosis can be mouse strain dependent.

MATERIALS AND METHODS

Strains and media. Strain B-5233 and its derivatives were maintained as previously described (37). RPMI 1640 alone or buffered with 25 mM HEPES, pH 7.2 (RPMI/HEPES), was used to grow the fungal strains in liquid cultures.

Deletion of *gliP* and complementation of the deletant strain. The deletion vector was constructed by inserting the hygromycin resistance cassette between a 1,070-bp fragment upstream and a 1,066-bp fragment downstream of the coding region of the gene *gliP* and cloned into the vector pDHt/SK2 (33). The deletion vector was integrated into the B-5233 genome via *Agrobacterium tumefaciens*-mediated transformation (33). Southern hybridization identified a transformant (referred as *gliP*Δ) with a homologous gene replacement resulting in deletion of *gliP*. To reconstitute *gliP*, a 7.6-kb fragment including 562 bp upstream and 603 bp downstream of the *gliP* coding region was integrated into the *gliP*Δ strain using *A. tumefaciens*-mediated transformation (33). Southern hybridization identified a strain (referred to as $gliP_R$) with a single inserted copy of the *gliP* gene.

CFs. The culture filtrates (CFs) used in the assays with EL4 thymoma cells and mouse embryonic fibroblasts (MEF) were prepared by inoculating 1×10^7 conidia in 100 ml of RPMI 1640 and incubating them for 48 h at 37°C and 5% CO_2. The CFs were diluted to 2.5, 5, 10, and 20% in RPMI 1640. CFs for neutrophil chemiluminescence assays and gliotoxin quantitation were prepared by inoculating 3×10^8 conidia in 100 ml of RPMI/HEPES and incubating them for 72 h at 37°C and 5% CO_2.

Quantitation of gliotoxin. The CFs were extracted with an equal volume of chloroform. The organic phase was recovered, dried, and dissolved in chloroform at 1/20 of the volume and dried again. The residue was dissolved in 100 μl of methanol and centrifuged at 14,000 × *g* for 5 min. High-performance liquid chromatography (HPLC) analysis was performed as described previously (2). The gliotoxin concentration was quantified using a standard curve of the pure compound (Sigma, St. Louis, MO). The column effluent was monitored at a wavelength of 275 nm.

Apoptosis/cell death of EL4 thymoma cells. Thymoma cells were chosen for apoptosis study because they are a good model for apoptotic markers (25). Preliminary experiments showed that commercially available gliotoxin effectively induces apoptosis in EL4 thymoma cells (J. Pardo and M. M. Simon, unpublished data). EL4 cells (2×10^5) in 1 ml of minimum essential medium supplemented with 5% fetal calf serum (FCS) were incubated for 16 h with 2.5, 5, 10, and 20% (vol/vol) CFs and analyzed for the following apoptotic parameters. The conformational change in the protein Bak was analyzed by fluorescence-activated cell sorter (FACS) using a polyclonal antibody (Ab) against the N-terminal sequence as previously described (26). Briefly, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% saponin in phosphate-buffered saline (PBS)-5% FCS, and incubated with 2 μg/ml rabbit polyclonal anti-Bak (NT; Upstate Biotechnology) or 5 μg/ml rabbit purified immunoglobulin G (control). After being washed with saponin, the cells were incubated with Alexa 647 goat anti-rabbit Ab, washed again, and analyzed by FACS. To monitor intracellular activation of caspase 3, the cells were fixed with 2.5% PFA, incubated with a fluorescein isothiocyanate-labeled monoclonal Ab against the active form of caspase 3 (clone C92605; BD Biosciences), and analyzed by FACS. Immunoglobulin G isotype antibody (BD Biosciences) was used as a control (25). Loss of mitochondrial-membrane potential and superoxide anion production were monitored with the fluorescent probe $DiOC_6[3]$ or dihydroethidium (Molecular Probes), respectively (25). Phosphatidylserine translocation and propidium iodide (PI) uptake were analyzed by FACS as described previously using the Annexin V Detection Kit (BD PharMingen, San Diego, CA) (25). Cells positive for Annexin V (AV) and PI and cells positive for AV but negative for PI were scored, and the sum of both was expressed as a percentage of the total population. In each experiment, 10^4 cells were counted, and specific cell death was obtained with the following formula: percent $AV^+/PI^+ + AV^+/PI^-$ cells = (percent $AV^+/PI^+ + AV^+/PI^-$ in treated cells) − (percent $AV^+/PI^+ + AV^+/PI^-$ in nontreated control). The proportion of AV^+/PI^+ plus AV^+/PI^- cells in controls was always lower than 15%.

Cell detachment of MEF. MEF transformed with simian virus 40 (26, 40) were cultured overnight in minimum essential medium supplemented with 10% FCS and 10^{-5} M 2-mercaptoethanol at 37°C and 7% CO_2. The medium was replaced by 2.5, 5, 10, and 20% CF (vol/vol), and the cells were monitored for cell detachment after 4 h of incubation (26, 40). Photographs were taken with a Zeiss Axiovert 10 and Axiovision software.

Inhibition of the oxidative burst in neutrophils. Human blood from healthy donors was collected by the Clinical Center Department of Transfusion Medicine and anticoagulated with acid dextrose citrate. Neutrophils were purified as described previously (42). The preparations were 95.6% ± 3% pure. Twenty-five microliters of RPMI/HEPES alone (control) or 20 μl of RPMI/HEPES plus 5 μl of the CFs were added to the wells of a white polypropylene 96-well plate (Whatman UNIPLATE). Neutrophils at 1×10^6/ml in RPMI/HEPES were added to each well (25 μl/well) and incubated at 37°C and 5% CO_2. After 30 min, the cells were quickly mixed with prewarmed 2× phorbol 12-myristate 13-acetate (PMA) diluted in the Diogenes chemiluminescent enhancer (National Diagnostics, Atlanta, GA), and relative light units (RLU) were measured for 0.5 s per well every 90 s in an Anthos Zenyth 3100 luminometer at 37°C with intermittent shaking. Raw data are presented as Sum RLU (equaling the sum of all the measurements of RLU over the first 60 min).

Virulence studies. All animal studies were approved by the institutional animal care and use committee. Ten- to 11-week-old male mice of the BALB/c and 129/Sv strains were immunosuppressed by subcutaneous injection of 2 mg hydrocortisone acetate in 100 μl PBS-0.1% Tween 20 on days −4, −2, 0, 2, and 4. On day zero, the mice were inoculated intranasally with 5×10^6 conidia in 20 μl of PBS-0.01% Tween 20. Nine mice per group of the BALB/c strain and 10 mice per group of the 129/Sv strain were used. Morbidity/mortality were monitored for up to 40 days, and the Kaplan and Meier survival with log rank test was used for comparisons among the groups. Control mice were immunosuppressed with hydrocortisone and inoculated intranasally with 20 μl PBS-0.01% Tween 20. Histopathological sections of the lungs of 129/Sv mice were prepared from one mouse from each group infected with B-5233 and *gliP*Δ and sacrificed at 72 and 96 h postinfection. The tissue sections were stained with hematoxylin and eosin or Gomori's methenamine-silver (GMS).

RESULTS

Deletion of *gliP*. The proposed pathway for the synthesis of gliotoxin starts with the condensation of the amino acids phenylalanine and serine by a nonribosomal peptide synthase encoded by the gene *gliP* (12). We deleted the *gliP* gene in the *A. fumigatus* strain B-5233 and observed that except for a slight tendency to produce more aerial hyphae, the *gliP*Δ strain did not show any morphological differences from strain B-5233. Growth in liquid media showed similar germination rates, as well as hyphal development, in the B-5233 and *gliP*Δ strains (data not shown). In the HPLC analysis, a peak with a retention time correlating with that of pure gliotoxin (approximately 42 min) was identified in the CFs from strain B-5233 and the

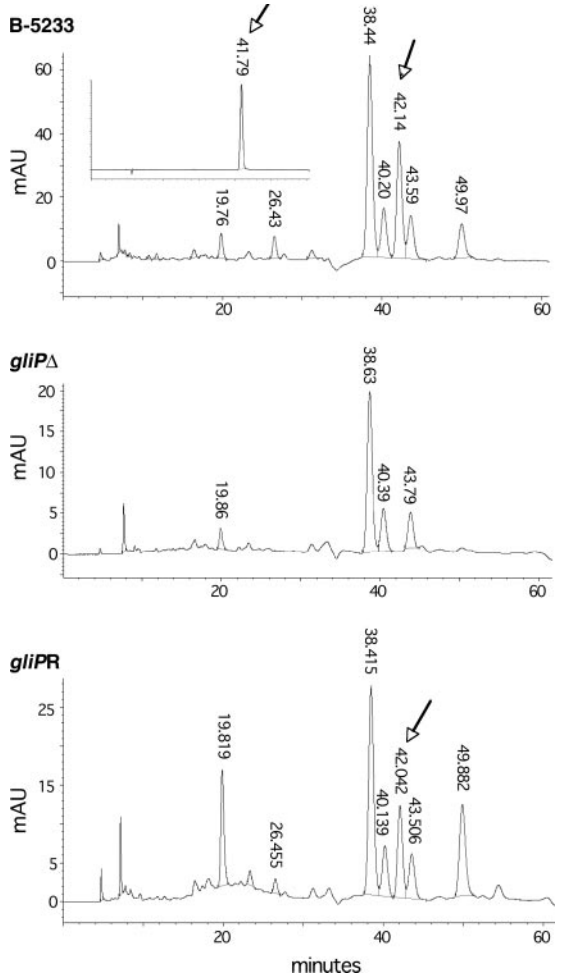

FIG. 1. HPLC analysis of gliotoxin in the CFs from strains B-5233, *gliP*Δ, and *gliP*$_R$. The arrows indicate the gliotoxin peaks. The inset in the first panel shows the gliotoxin standard. A representative HPLC analysis is shown. mAU, milliabsorbance units.

gliP$_R$ strain (the reconstituted strain), but not from the *gliP*Δ strain, confirming that deletion of *gliP* abolished synthesis of gliotoxin (Fig. 1). Two extra peaks (26 and 49 min) were identified in the CFs from strain B-5233 and the *gliP*$_R$ strain. The identities of these peaks are unknown; however, since the peaks were detected only in the CFs from strain B-5233 and the *gliP*$_R$ strain, it is possible that they represent intermediate metabolites in gliotoxin synthesis. Quantitative HPLC analysis showed that the CFs from strain B-5233 and the *gliP*$_R$ strain had similar concentrations of gliotoxin, 243.1 ± 64.4 ng/ml and 183.1 ± 9.5 ng/ml. The data are given as a mean ± standard error of the mean (SEM) from three independent experiments.

Apoptosis/cell death of EL4 thymoma cells. Considering the proapoptotic activity of purified gliotoxin (26), the CFs from the B-5233, *gliP*Δ, and *gliP*$_R$ strains were evaluated for their proapoptotic potentials. Incubation of the EL4 cells with the CFs from strains B-5233 and *gliP*$_R$, but not *gliP*Δ, resulted in conformational changes of Bak and subsequent production of superoxide anions, caspase 3 activation, and mitochondrial depolarization in a dose-dependent manner (Fig. 2A to D). Superoxide anions were detected in over 50% of the cells incubated with 10% or a higher percentage of CFs from strains B-5233 and *gliP*$_R$, but the anions were not detected when the cells were incubated with *gliP*Δ CFs at any of the concentrations tested (Fig. 2B). Furthermore, caspase 3 activity and mitochondrial depolarization were detected in more than 75% of the cells treated with 10% or higher concentrations of CFs from strains B-5233 and *gliP*$_R$, but not from *gliP*Δ CFs (Fig. 2C and D). The EL4 cells were also monitored for phosphatidylserine translocation using AV and for membrane lysis using PI (Fig. 2E). At 10% or higher concentrations of CFs from strain B-5233 or *gliP*$_R$, over 75% of the cells were either dead or in the late stages of apoptosis. In contrast, neither phosphatidylserine exposure nor incorporation of PI was observed in the cells incubated with *gliP*Δ CFs. Addition of gliotoxin (1 μM) to the *gliP*Δ CFs resulted in an induction of apoptosis similar to that observed with B-5233 CFs (data not shown).

Phosphatidylserine translocation was also observed in bone marrow-derived mouse neutrophils incubated with the CFs from the wild type, but not with the CFs from the *gliP* mutant (data not shown). These results suggest that the gliotoxin in the CFs of strains B-5233 and *gliP*$_R$ is a critical component for the induction of apoptotic events and cell death.

Cell detachment of MEF. The CFs were tested for their ability to induce detachment of anchorage-dependent cells, a process known to be elicited by gliotoxin (20). Whereas untreated MEF grown in RPMI remained adhered to the surface (control), addition of as little as 2.5% CFs from either strain B-5233 or *gliP*$_R$ caused some of the MEF to display a rounded shape (Fig. 3). At 5% or higher concentrations of CFs, a majority of the cells had a rounded shape and were detached from the surface. In contrast, *gliP*Δ CFs did not cause detachment even at higher concentrations (Fig. 3), suggesting that gliotoxin is involved in MEF detachment.

Inhibition of the oxidative burst in human neutrophils. Gliotoxin affects host defenses in several ways, including inhibition of the myeloid NADPH oxidase (38), the enzyme that produces antimicrobial oxygen radicals. A chemiluminescent reagent system that produces light in response to superoxide anions was used to monitor the PMA-induced oxidative burst of neutrophils incubated with CFs. PMA induces a dose-dependent chemiluminescence in neutrophils incubated with RPMI/HEPES (data not shown). Preincubation of the neutrophils with 5% CFs from strains B-5233 and *gliP*$_R$, however, resulted in a strong decrease in chemiluminescence without affecting the viability of the neutrophils (Fig. 4 and data not shown). In contrast, preincubation of neutrophils with *gliP*Δ CFs resulted in a PMA-induced chemiluminescence that was comparable to that of the RPMI control, although slightly lower (Fig. 4). Spiking the *gliP*Δ CFs with pure gliotoxin significantly reduced the chemiluminescence of the neutrophils, similarly to that observed with B-5233 CFs (data not shown). Control experiments using xanthine and xanthine oxidase as noncellular sources of superoxide anions demonstrated that the CFs did not quench detection of the superoxide in this assay (data not shown).

Virulence. Since the CFs from the *gliP*Δ strain showed reduced ability to induce mammalian-cell death and caused significantly less inhibition of the neutrophil oxidative burst, we evaluated the virulence of this mutant in murine models. The mouse strains 129/Sv and BALB/c were immunosuppressed

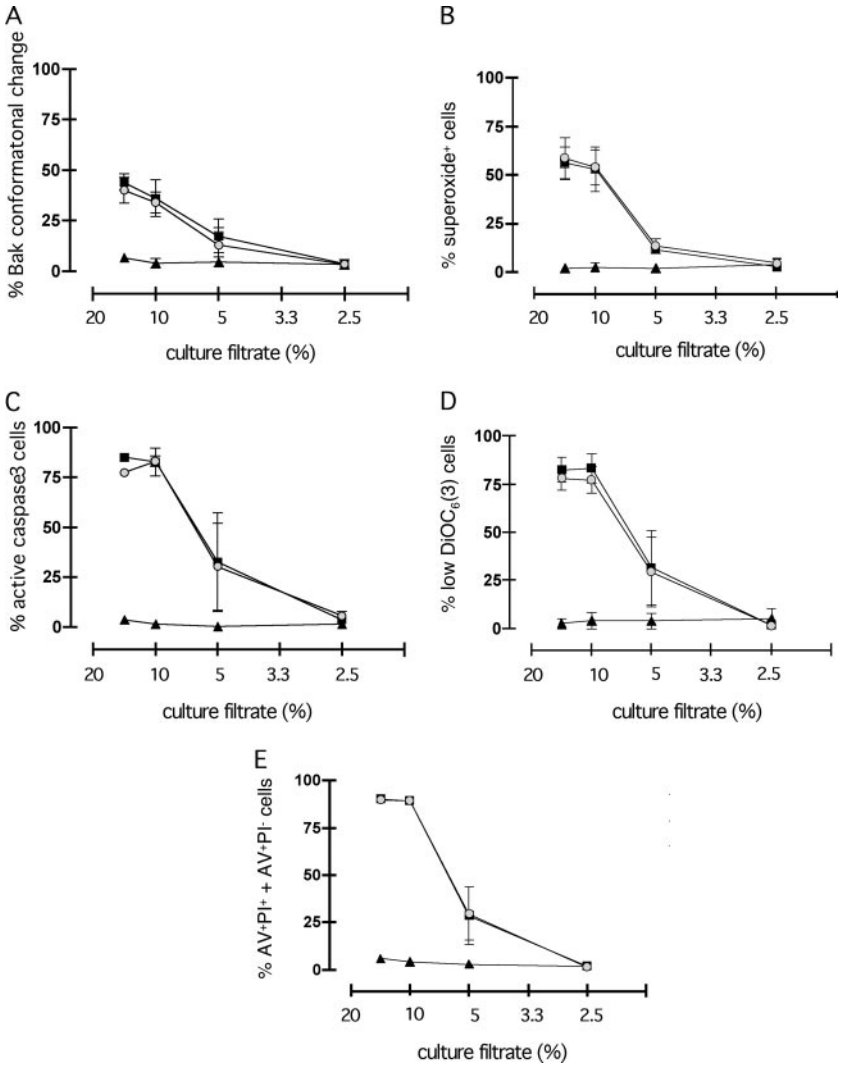

FIG. 2. Apoptosis and cell death of EL4 thymoma cells. EL4 cells were incubated for 18 h with diluted CFs from strains B-5233 (squares), *gliP*Δ (triangles), and *gliP*$_R$ (circles). The dilutions of CFs tested were 2.5, 5, 10, and 20% (vol/vol). (A) Bak conformational changes were analyzed by FACS using a rabbit polyclonal antibody against the N-terminal sequence (NT; Upstate) and Alexa 647 goat anti-rabbit Ab. (B) Production of superoxide anions was tested with 2-hydroxiethidium staining. (C) Caspase 3 activation was monitored by FACS using a monoclonal Ab against the active enzyme. (D) The mitochondrial-membrane potential was monitored with $DiOC_6$[3] staining. (E) Membrane integrity was tested by phosphatidylserine translocation (AV) and membrane lysis (PI). The data are given as means ± SEM from three independent experiments.

with hydrocortisone acetate and infected with *Aspergillus* strains. With 129/Sv mice, a clear difference was seen in the survival rates between the groups infected with strains B-5233 and *gliP*Δ ($P = 0.005$). Fifty percent of the mice infected with strain B-5233 or *gliP*$_R$ died within 19 days, whereas only 22% of the mice infected with the *gliP*Δ strain died in the same period (Fig. 5A). At day 40 postinfection, approximately 80% of the mice infected with the *gliP*Δ strain were still alive. These results suggest that the deletant strain is less virulent than the wild type. Histological analysis of lung sections confirmed the reduced virulence of the *gliP*Δ strain. Lung sections prepared 72 h postinfection with strain B-5233 showed multifocal bronchopneumonia with necrosis, neutrophilic infiltration, and airways filled with necrotic debris (Fig. 5C) and hyphae. In affected bronchioles, there was full-thickness necrosis of the walls extending from the epithelium through the surrounding smooth muscle into the adjacent pulmonary parenchyma (data not shown). In GMS-stained sections, hyphae were observed within as well as outside the bronchioles. By 96 h, hyphae of the B-5233 strain were widely scattered throughout the lung and the pulmonary architecture was effaced by zones of necrosis and inflammation with hemorrhage and edema (Fig. 5E and data not shown). The bronchopneumonia observed in the lungs of mice 72 h postinfection with the *gliP*Δ strain was much less severe than that seen in B-5233-infected mice (Fig. 5D). The lesions caused by the *gliP*Δ strain were more discreet and often spared the bronchiolar epithelium at 72 h and 96 h (data not shown). At 96 h, unlike the lungs infected by the wild type, hyphae of the *gliP*Δ strain were still confined primarily to the bronchioles (Fig. 5F). In comparisons with the wild-type strain, progression of the pulmonary lesions caused by *gliP*Δ infection in the sections from three different parts of the lungs from a

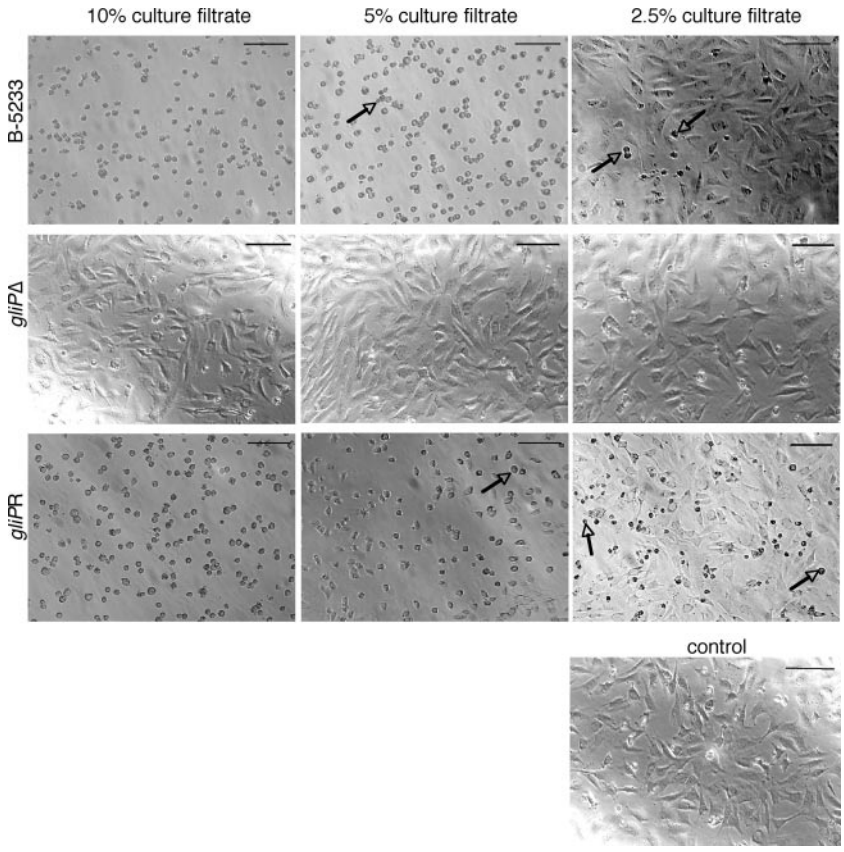

FIG. 3. Cell detachment of MEF. MEF were incubated for 4 h with 2.5, 5, and 10% (vol/vol) CFs. The cells incubated with RPMI 1640 (control) were flat and elongated, forming a monolayer adhering to the surface. When the MEF were incubated with 2.5% CFs from strain B-5233 or *gliP*$_R$, some cells displayed a rounded shape (arrows). Incubation with 5 and 10% CFs from strain B-5233 or *gliP*$_R$ caused a majority of the cells to become round and lose anchorage. Changes in MEF morphology or detachment were not observed at any concentration of *gliP*Δ CFs. Bar, 100 μm.

mouse in each group was appreciably delayed, which corroborates the survival data. It is possible that lesions in the mice infected with the *gliP*Δ strain that survived for 40 days, when the experiment was terminated, may never have progressed beyond the bronchiole. Further histopathological studies with more mice from each group will confirm our observations.

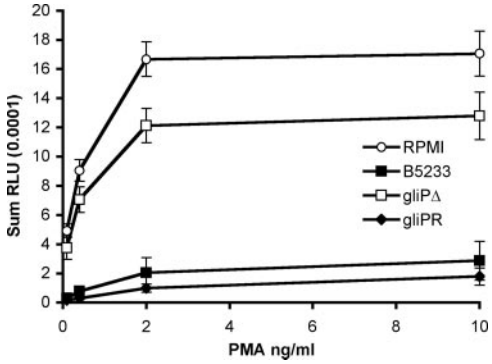

FIG. 4. Chemiluminescence of neutrophils incubated with CFs from strains B-5233, *gliP*Δ, and *gliP*$_R$. The cells were incubated in 5% CFs (vol/vol) for 30 min before PMA was added to activate the NADPH oxidase. The data shown represent the means of Sum RLU (±SEM; n = four to six donors).

Compared to 129/Sv mice, BALB/c mice appeared to be more susceptible to *A. fumigatus*; 50% of the group infected with strain B-5233 died within 12 days postinfection. In the group infected with the *gliP*Δ strain, the 50% death rate was reached at 18 days postinfection (Fig. 5B). The difference in the survival rates showed that the *gliP*Δ strain is less virulent than B-5233 ($P = 0.007$), as had been observed with the 129/Sv mice. The mortality of the group infected with the *gliP*$_R$ strain was similar to that observed with strain B-5233. The significantly lower mortality rate of mice infected with the *gliP*Δ strain compared to strains B-5233 and *gliP*$_R$ in the two genetically distinct mouse strains suggests that gliotoxin is an important virulence determinant of *A. fumigatus*.

DISCUSSION

Recent studies have exposed the multiple immunosuppressive properties of gliotoxin, including inhibition of phagocytosis by macrophages (9, 19), activation of NF-κB (23), mast cell activation (21), and induction of apoptosis in various cells of the immune system (30, 32, 34). Gliotoxin-induced apoptosis was demonstrated in peripheral blood mononuclear cells and macrophages (30, 32) in vitro and in cells of the thymus, spleen, and mesenteric lymph nodes in mice challenged with a lethal dose of gliotoxin (35). Although it is well known that

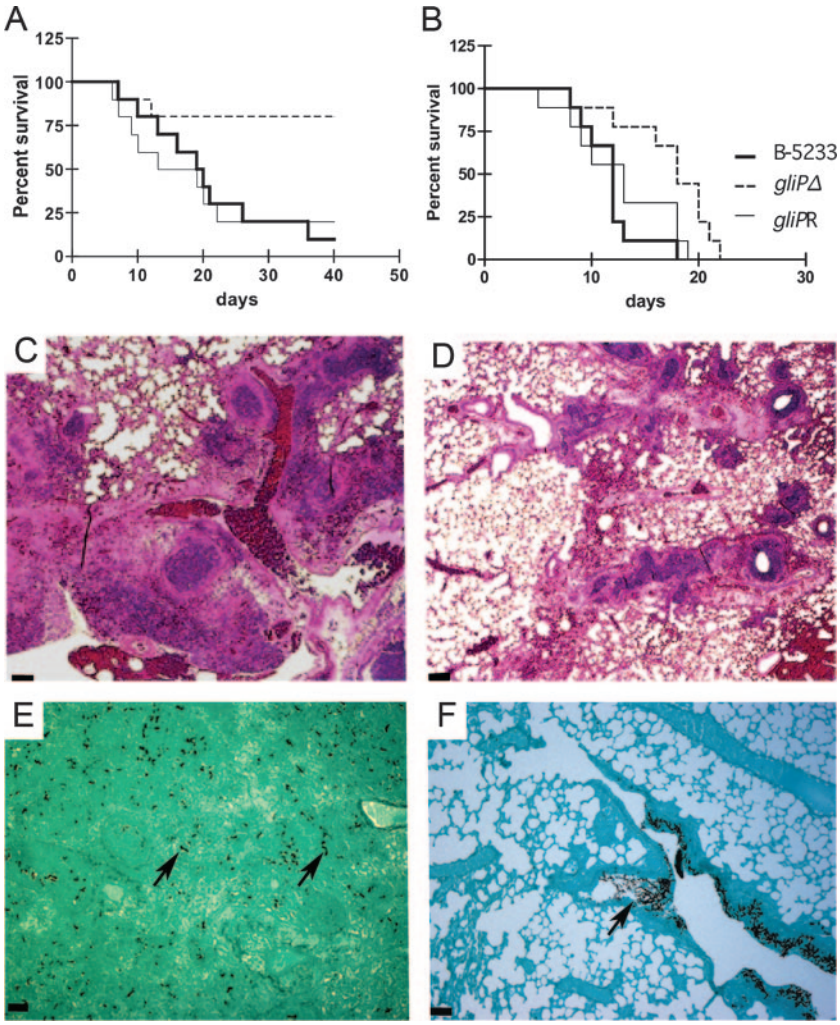

FIG. 5. Virulence studies with the mouse strains 129/Sv and BALB/c. Mice were immunosuppressed with hydrocortisone and inoculated intranasally with 5×10^6 conidia. The survival rates of the recipient 129/Sv and BALB/c mice are shown in panels A and B, respectively. The Kaplan and Meier survival with log rank test was used for comparisons of survival levels among the groups: comparison between the groups infected with strains B-5233 and *gliP*Δ showed a *P* value of 0.005 for 129/Sv mice and a *P* value of 0.007 for BALB/c mice. The virulence assay with the 129/Sv strain was repeated three times with similar results. (C and D) Hematoxylin and eosin-stained lung sections prepared 72 h postinfection of 129/Sv mice with strain B-5233 (C) or *gliP*Δ (D). (E and F) GMS-stained lung sections prepared 96 h postinfection with strains B-5233 (E) and *gliP*Δ (F). The arrows indicate hyphae. Bar, 20 μm.

gliotoxin induces apoptosis in mammalian cells, it was only recently that the detailed mechanism(s) underlying these processes was elucidated (26). It was shown that the gliotoxin-induced apoptotic cell death in MEF was initiated by direct activation of the proapoptotic Bcl-2 family member Bak, followed by generation of reactive oxygen species (ROS). The ROS facilitated the release of cytochrome *c* and apoptosis-inducing factors from mitochondria, leading to caspase activation, as well as other events that mediate cell death (26). Similar to the studies with pure gliotoxin, we showed that *Aspergillus* CFs containing gliotoxin (CFs from strain B-5233 or $gliP_R$) induced Bak conformational changes in EL4 thymoma cells, which were accompanied by the production of superoxide anions (ROS), mitochondrial depolarization, capase 3 activation, and apoptotic cell death. The CFs lacking gliotoxin (from the *gliP*Δ strain), however, did not induce activation of Bak or any other features of the mitochondrial apoptotic process. A similar apoptotic cell death in mouse neutrophils was observed with the wild-type CFs and not with the *glip*Δ CFs (data not shown). These results suggest that the described proapoptotic events that occur in vitro in response to pure gliotoxin may similarly occur in the host infected with *A. fumigatus*.

A comparison of the biological activities of three mycotoxins produced by *A. fumigatus*, namely, fumagillin, helvolic acid, and gliotoxin, showed that only gliotoxin suppressed O_2^- generation in neutrophils (38). It has been suggested that gliotoxin inhibits the respiratory burst by targeting the assembly of the NADPH oxidase complex (38) and affecting an essential site(s) for electron transport in the flavocytochrome b558 (22). Our results showed that only the strains producing gliotoxin, strains B-5233 and $gliP_R$, were able to strongly inhibit the generation of superoxide anions in PMA-stimulated neutrophils (Fig. 4). Furthermore, when the CFs lacking gliotoxin were supplemented with pure toxin, the inhibitory effect on the oxidative

burst of neutrophils was restored to wild-type levels. The evidence provided here suggests that *A. fumigatus* uses gliotoxin to evade the production of ROS in vivo. Whether gliotoxin is the only metabolite possessing such inhibitory properties is unknown.

Recent reports from three different laboratories have shown that the elimination of gliotoxin synthesis in the AF293 strain did not affect virulence in BALB/c and ICR mice immunosuppressed with cyclophosphamide and cortisone acetate (3, 5, 14). In two of these studies, it was suggested that the immunosuppressive regimen used in the virulence assays, a combination of cyclophasphamide and cortisone, may have been inadequate to show the reduction in virulence by gliotoxin-negative mutants and needed less traumatic immunosuppression (3, 14). One of the most important experimental factors with aspergillosis in the mouse model is the immune status of the mice. Typically, the immunosuppressive regimens used in mouse models for IA rely on cyclophosphamide and/or cortisone. Cyclophosphamide is a broad immunosuppressive agent affecting both innate and adaptive cellular components (10). This drug is used to deplete neutrophils and to render mice susceptible to the development of IA (28, 31). Corticosteroids are also broadly immunosuppressive, but their primary mechanism of action targets the alteration of the function of immune cells rather than depleting them (10). It has been shown that immunosuppression with hydrocortisone impairs neutrophil-mediated damage of *A. fumigatus* hyphae (27). Thus far, there is no consensus on the suitability of immunosuppressive regimens for experimental IA. Dixon and collaborators demonstrated that cyclophosphamide and cortisone acetate can be used separately to cause immunosuppression in mouse models for IA (7). For instance, the investigation of the role of a catalase in *A. fumigatus* used cortisone acetate as the immunosuppressive drug (4). In other studies, such as those that involved the determination of the virulence of *A. fumigatus* mutant strains with deletion of the genes *rhbA* (24), *pkaC1*, and *gpaB* (18), mice were immunosuppressed with a combination of cyclophosphamide and cortisone acetate. An important observation by Balloy and collaborators showed that the infection patterns and the types of inflammation caused by *A. fumigatus* differed between mice immunosuppressed with corticosteroids and other chemotherapeutics (1).

As gliotoxin inhibits the neutrophil's oxidative burst, abrogation of this mycotoxin from *A. fumigatus* is likely to reduce the ability of the fungus to survive a neutrophil attack. However, if the mice are neutropenic, as is the case with mice imunosuppressed with cyclophosphamide, gliotoxin may not be as important for *A. fumigatus* to survive inside the host, and therefore, the *gliP*Δ strain can be as virulent as the wild-type strain. Based on this assumption, we reasoned that the mice immunosuppressed with both cyclophosphamide and cortisone acetate in previous studies might have been excessively immunosuppressed and the differences in virulence between the wild type and the gliotoxin-negative mutant was not detectable. Therefore, we chose to immunosuppress two different mouse strains, 129/Sv and BALB/c, with hydrocortisone alone for this study. In contrast to the published studies (3, 5, 14), we found that the *gliP*Δ strain is significantly less virulent than the wild type or the $gliP_R$ strain in both mouse strains, and the degree of attenuation in virulence was even more significant in 129/Sv than in BALB/c mice. A result corroborating ours was also obtained with BALB/c mice immunosuppressed with cortisone acetate using the AF293 strain and its *glip*Δ strains (G. May, personal communication). These results support the hypothesis that gliotoxin is an important determinant of *A. fumigatus* virulence and indicate that an appropriate immunosuppressive regimen is required to reveal the role of gliotoxin in the pathogenesis of IA. Our results also suggest that the host genetic background is an important factor in the susceptibility of mice to *A. fumigatus*. In fact, it has been reported that factors such as genetic background and polymorphism in the genes regulating innate and adaptive immune responses are likely to contribute to the susceptibility of mice to fungal infections (41).

In conclusion, deletion of the *gliP* gene in *A. fumigatus* strain B-5233 resulted in abrogation of gliotoxin synthesis, failure to induce apoptosis in mammalian cells, and a reduced ability to inhibit the oxidative burst in human neutrophils in vitro. Most importantly, the *gliP*Δ mutant showed reduced virulence in two different mouse strains, indicating that, contrary to previous reports (3, 5, 14), gliotoxin is a virulence determinant of *A. fumigatus*.

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