The Initial 96 Hours of Invasive Pulmonary Aspergillosis: Histopathology, Comparative Kinetics of Galactomannan and (1→3)-β-D-Glucan, and Consequences of Delayed Antifungal Therapy

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Acute invasive pulmonary aspergillosis is a rapidly progressive and frequently lethal infection. Relatively little is known about early events in the pathogenesis and relationship between the cell wall biomarkers galactomannan and (1→3)-β-D-glucan. The consequences of delayed antifungal therapy are also poorly defined. A persistently neutropenic rabbit model of invasive pulmonary aspergillosis was used to describe the histopathology of early invasive pulmonary aspergillosis and the kinetics of galactomannan and (1→3)-β-D-glucan. The time course of both molecules was mathematically modeled by using a population methodology, and Monte Carlo simulations were performed. The effect of progressive delay in the administration of amphotericin B deoxycholate 1 mg/kg at 24, 48, 72, and 96 h postinoculation on fungal burden, lung weight, pulmonary infarct score, and survival was determined. Histopathology showed phagocytosis of conidia by pulmonary alveolar macrophages at 4 h postinoculation. At 12 to 24 h, there was a progressive focal inflammatory response with conidial germination and hyphal extension. Subsequently, hyphae invaded into the contiguous lung. Galactomannan and (1→3)-β-D-glucan had similar trajectories, and both exhibited considerable interindividual variability, which was reflected in Monte Carlo simulations. Concentrations of both molecules began to rise <24 h postinoculation before pulmonary hemorrhagic infarction was present. Delays of 72 and 96 h in the administration of amphotericin B resulted in fungal burdens and lung weights that were indistinguishable from those of controls, respectively. Galactomannan and (1→3)-β-D-glucan have similar kinetics and are comparable biomarkers of early invasive pulmonary aspergillosis. Antifungal treatment at ≥48 h postinoculation is associated with suboptimal therapeutic outcomes.

Acute invasive pulmonary aspergillosis is a leading cause of morbidity and mortality in immunocompromised patients. There have been considerable efforts to improve the diagnostic accuracy and therapeutic outcomes associated with this frequently lethal infection. A better understanding of the relationship of clinically relevant biomarkers and the pathogenesis of invasive pulmonary aspergillosis would facilitate further development of strategies to identify and treat patients at the earliest possible time.

Galactomannan and (1→3)-β-D-glucan are complex carbohydrate cell wall molecules produced by *Aspergillus* spp. The diagnostic and prognostic value of these biomarkers in experimental models and humans is relatively well characterized (7, 12, 17, 18, 23). Despite this, there is little understanding of the relationship of the time course of galactomannan and (1→3)-β-D-glucan to early events in the pathogenesis of invasive pulmonary aspergillosis, and no studies that have rigorously compared their kinetics in laboratory animals or humans.

Recently, we described the kinetics of galactomannan in persistently neutropenic rabbits with early invasive pulmonary aspergillosis (12). In the present study, we extend our previous findings by defining the critical histopathological events in early invasive pulmonary aspergillosis and further characterize and compare the kinetics of the circulating fungal antigens galactomannan and (1→3)-β-D-glucan. We further explore the consequences of delayed antifungal therapy with amphotericin B deoxycholate to provide insight into the period after inoculation in which favorable therapeutic outcomes can be secured in profoundly immunocompromised hosts.

**MATERIALS AND METHODS**

**Organism.** A well-characterized isolate of *Aspergillus fumigatus*, NIH 4215 (ATCC MYA-1163), was used for all experiments. The MIC of amphotericin B, as determined by using Clinical and Laboratory Standards Institute methodology (3), was 0.5 mg/liter.

**Rabbit model of invasive pulmonary aspergillosis.** A well-described (19) persistently neutropenic rabbit model of invasive pulmonary aspergillosis was used for all experiments. Briefly, female New Zealand White rabbits weighing 2.6 to 3.5 kg were used. The care of the rabbits was conducted according to the guidelines of the Association for Assessment and Accreditation of Laboratory
Animal Care and the approved by the Animal Care and Use Committee of the National Cancer Institute. Rabbits were individually housed, and food and water were provided ad libitum. All rabbits had an indwelling intravenous catheter placed under general anesthesia; this enabled the atraumatic acquisition of diagnostic samples and parental administration of agents required to induce and support neutropenia, as well as amphotericin B deoxycholate for the experiment examining the effect of therapeutic delay.

Rabbits were immunosuppressed with cytarabine (cytosine arabinoside; Cytostar-U; Pharmacia-Upjohn, Kalamazoo, MI) and methylprednisolone, as previously described. Cytarabine was administered daily (intravenously [i.v.]), 525 mg/m² beginning on day −1 relative to inoculation. Two dosages of methylprednisolone were administered on days −1 and 0. Vancomycin, ceftazidime, and gentamicin were administered i.v. daily to prevent opportunistic bacterial infections. Rabbits were inoculated via the endobronchial instillation of 3 × 10⁶ conidia in a 0.25-ml volume, as previously described (19).

Studies of early invasive pulmonary aspergillosis. The histopathology and kinetics of galactomannan and (1→3)-β-D-glucan were determined over the course of three separate experiments, and the entire data set was pooled for analysis and population modeling. Serum samples for galactomannan and (1→3)-β-D-glucan concentrations were collected from the indwelling catheter throughout the experimental period. Rabbits (n = 4 per group) were sequentially sacrificed 4, 8, 12, 16, 20, 24, 36, 48, 72, and 96 h postinoculation by the injection of 65 mg of pentobarbital (Scherling Plough, Union, NJ). The mediastinal structures were removed en bloc; subsequently, the lungs and trachea were dissected free and weighed. A bronchoalveolar lavage was performed with 20 ml of 0.9% saline, and the retrieved fluid was submitted for quantitative culture and estimation of galactomannan concentrations. Representative sections were taken from each lobe, weighed, homogenized, and submitted for quantitative culture.

Galactomannan and (1→3)-β-D-glucan. The concentration of galactomannan in serum and bronchoalveolar lavage fluid was determined by using a commercially available double sandwich enzyme-linked immunosorbent assay (Bio-Rad, Marnes la Coquette, France) according to the manufacturer’s instructions. Similarly, the concentration of (1→3)-β-D-glucan in serum was determined by using the experimental assay according to the manufacturer’s instructions (Associates of Cape Cod, Inc., Falmouth, MA). To determine the median time to positivity, a cutoff value of 0.5 and 60 pg/ml was used for galactomannan and (1→3)-β-D-glucan, respectively. This analysis was limited to rabbits that had data for both antigens at every time point.

Studies of delayed antifungal therapy. Three separate experiments were performed. Rabbits (n = 10 per group) received amphotericin B deoxycholate 1 mg/kg daily, given i.v. via the central venous catheter. The initiation of therapy was delayed for 24, 48, 72, and 96 h postinoculation. Therapy was continued until death or the end of the study (312 h postinoculation). At the time of death, the lungs were removed and weighed, and the number of pulmonary infarcts was counted. Lung weight and infarct score are measures of organism-mediated pulmonary injury. The infarct score is determined by the number of lobes with hemorrhagic infarcts per rabbit (maximum of six/rabbit) and is not a measure of the severity of a given infarct. Representative sections from each lobe were submitted for quantitative culture.

Statistics, mathematical modeling, and Monte Carlo simulations. The time course of serum galactomannan and (1→3)-β-D-glucan concentrations was modeled by using a population methodology. Population modeling was performed by using the Big version of the program Nonparametric Adaptive Grid (Big NPAG) (14). In these analyses, each rabbit was treated as an “individual” that contributed multiple samples; i.e., the data from each individual rabbit were modeled rather than the mean value for a group of rabbits. The following equation was used to model the kinetics of both galactomannan and (1→3)-β-D-glucan:

\[
dX(1) \, dX(1) = K_x \times \left(1 - \frac{X(1)}{POP\text{MAX}}\right) \times X(1)
\]

where \(K_x\) is the growth constant, and POPMAX is the theoretical maximum galactomannan or (1→3)-β-D-glucan concentration in the serum compartment \(X\) (1). As the concentration of either antigen approaches POPMAX, growth slows and then ceases.

The data were weighted by the inverse of the estimated variance for the respective assays; this was obtained by running samples with a range of concentrations in triplicate. The fit of the model to the data was assessed by visual inspection of the observed-predicted values after the Bayesian step and the coefficient of determination \(r^2\) of the linear regression of the observed-versus-predicted values, along with measures of bias (mean weighted error) and precision (bias-adjusted mean weighted squared error).

To compare the concentration-time profiles of galactomannan and (1→3)-β-D-glucan, the correlation between paired measurements were assessed. Furthermore, the area under the concentration-time curve (AUC₀⁻∞) for galactomannan and (1→3)-β-D-glucan in each rabbit was estimated by integration using the program ADAPT II (6). The Bayesian estimates for the parameter values that described the concentration-time profile for individual rabbits were obtained. Monte Carlo simulation was then used to further explore the implications of the experimental data and the mathematical model. These simulations were performed using the pharmacokinetic program ADAPT II (6). The estimates for central tendency for each of the parameters [mean and median for (1→3)-β-D-glucan and galactomannan, respectively] and their variances were inserted into subroutine PRIOR of ADAPT II (6). A series of 9,999-rabbit simulations were performed. Both normal and log-normal parameter distributions were evaluated and assessed for their ability to recapitulate the initial parameter values and their distributions. The trajectories for the 2.5th, 50th, and 97.5th percentiles for the simulated population were plotted to provide a further assessment of the extent of interindividual variability.

The survival of rabbits treated after various delays was assessed by using a Kaplan-Meier model implemented within the statistical program SYSTAT version 11. Rabbıts that survived to the end of the study were right-censored. The correlation between paired concentration and the AUCs for galactomannan and (1→3)-β-D-glucan was assessed by using Spearman’s coefficient. Analysis of variance was used to compare the log₁₀(CFU/g) infect scores, and lung weights between the various treatment delay groups. A Bonferroni correction was used to adjust for multiple comparisons.

RESULTS

Histopathology. The histopathological appearances revealed a rapidly progressive infectious process. At 4 h postinoculation, few conidia were seen free within alveoli, while the majority had been phagocytosed by pulmonary alveolar macrophages (Fig. 1). The structure of the lung appeared normal at this early time point. Within the initial 8 to 24 h of infection, there were increasingly prominent focal areas of dense inflammatory infiltrates observed throughout the lung (Fig. 2). At 8 to 16 h
there was evidence of conidial germination and early hyphal formation. From 24 to 96 h there was progressive hyphal extension within inflammatory foci.

**Lung weight, quantitative fungal culture, and infarct score.** The rapidly progressive histopathological changes associated with hyphal extension and invasion were mirrored by an increase in lung weight, which increased in a linear manner in the first 96-hours of infection (coefficient of determination 0.96, \( P < 0.001 \); Fig. 3). Pulmonary infarction was first apparent \( \geq 24 \) h postinoculation. Subsequently, the number of infarcted lobes remained relatively constant for the remainder of the study period. There was no discernible temporal relationship for log10CFU/g in lung tissue and BAL. Somewhat paradoxically, the highest fungal burden was observed immediately postinoculation, after which there was a rapid decline to low (and in some cases undetectable) levels. There was no relationship between changes in log10CFU/g and the progressive histopathological appearances shown in Fig. 2.

**Galactomannan and (1\(\rightarrow\)3)-\(\beta\)-d-glucan concentrations.** The fit of the mathematical model to the data was acceptable for both fungal biomarkers. The population parameter median and mean values performed slightly better when obtaining Bayesian estimates for galactomannan and (1\(\rightarrow\)3)-\(\beta\)-d-glucan in each individual rabbit, respectively. The parameter values and their standard deviations are summarized in Table 1. The coefficients of determination after the Bayesian step were 0.83 and 0.89 for galactomannan and (1\(\rightarrow\)3)-\(\beta\)-d-glucan, respectively (Fig. 4). The measures of bias and precision for galactomannan were \(-0.30\) and 0.81, respectively, and for (1\(\rightarrow\)3)-\(\beta\)-d-glucan they were \(-0.2\) and 0.84, respectively.

Serum concentrations of (1\(\rightarrow\)3)-\(\beta\)-d-glucan and galactomannan increased above baseline values within the first 24 h of infection (Fig. 5). The concentrations of galactomannan were observed significantly earlier than those for (1\(\rightarrow\)3)-\(\beta\)-d-glucan (Fig. 6). The median time to positivity for galactomannan and (1\(\rightarrow\)3)-\(\beta\)-d-glucan was 12 and 30 h, respectively \(( P < 0.001 \). There was a rapid increase in the serum concentration of both biomarkers after becoming positive before a plateau was reached. The concentration of galactomannan in BAL was high throughout the study period, with no discernible relationship with serum concentrations (Fig. 5A).

The initial population parameter values describing the time...
course of galactomannan and (1→3)-β-D-glucan were recapitulated with a 9,999-rabbit Monte Carlo simulation. A log-normal parameter distribution was used for these simulations. The simulations revealed the extent variability in the trajectories of galactomannan and (1→3)-β-D-glucan that could be expected if the same experiment was conducted with 9,999 rabbits. As demonstrated in Fig. 5, the trajectory for (1→3)-β-D-glucan and galactomannan for an individual rabbit was highly variable. This variability was reflected in the estimates for the standard deviation for the parameter values [for both (1→3)-β-D-glucan and galactomannan; see Table 1] and was readily apparent from the Monte Carlo simulations, where there were considerable differences in the trajectories for the 2.5th, 50th, and 97.5th percentiles of the simulated population (Fig. 5B and D).

Paired measurements of galactomannan and (1→3)-β-D-glucan concentrations were positively correlated (Spearman coefficient, 0.761; 122 observations; \( P < 0.001 \); Fig. 7). The Bayesian estimates for the parameter values from each rabbit were used to calculate the area under the galactomannan and (1→3)-β-D-glucan concentration-time curve for that individual rabbit. The estimates for AUC\(_{0–96}\) for both galactomannan and (1→3)-β-D-glucan were positively correlated, but this did not reach statistical significance (Spearman coefficient, 0.266; 37 rabbits; \( P = 0.11 \); Fig. 7).

Effect of delay on therapeutic outcome. The impact of delayed therapy on the fungal burden (log\(_{10}\)CFU/g), infarct score, and lung weight is depicted in Fig. 8. A statistically

<table>
<thead>
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<th>Parameter(^a)</th>
<th>Mean</th>
<th>Median</th>
<th>SD</th>
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<td>0.074</td>
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<td>5.02</td>
<td>0.49</td>
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<tr>
<td>1,3-β-D-Glucan</td>
<td></td>
<td></td>
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<tr>
<td>( K_g )</td>
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<td>0.095</td>
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<td>6.14</td>
</tr>
<tr>
<td>POPMAX</td>
<td>522.63</td>
<td>403.94</td>
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\( K_g \) is the growth constant, the initial condition is the baseline reading immediately after inoculation, and POPMAX is the theoretical maximum concentration for the respective antigens.
significant decrease in these endpoints compared to controls was observed after delays of 24 and 48 h in the initiation of amphotericin B deoxycholate. Importantly, however, a delay in therapy of 72 h resulted in higher fungal burden and greater pulmonary injury, as measured by heavier lungs, than was observed with earlier therapy, to the extent that these values did not deviate in a statistically significant manner from controls. A delay in treatment of 72 h, however, did not affect the infarct score or the probability of survival (Fig. 8).

FIG. 4. Observed-versus-predicted values for galactomannan (A) and 1,3-β-D-(1→3)-β-D-glucan (B) after the Bayesian step following the fit of the mathematical model to the data from the respective fungal antigens.

FIG. 5. Kinetics of galactomannan and (1→3)-β-D-glucan in early invasive pulmonary aspergillosis. (A) Kinetics of galactomannan in serum (□) and bronchoalveolar lavage fluid (BAL; ■). (B) Trajectories for the 2.5th, 50th, and 97.5th percentiles for galactomannan from a population of 9,999 simulated rabbits with invasive pulmonary aspergillosis. (C) Kinetics of (1→3)-β-D-glucan in serum. (D) Trajectories for the 2.5th, 50th, and 97.5th percentiles for (1→3)-β-D-glucan from a population of 9,999 simulated rabbits with invasive pulmonary aspergillosis. The data are means ± the SEM.
DISCUSSION

Acute invasive pulmonary aspergillosis is a rapidly progressive life-threatening infection. Despite recent advances in a wide range of diagnostic and therapeutic modalities, invasive pulmonary aspergillosis remains a leading cause of morbidity and mortality for immunocompromised patients. The present analyses suggest that there is a surprisingly limited window available in which to secure a diagnosis and a favorable therapeutic outcome for invasive pulmonary aspergillosis in profoundly immunocompromised hosts.

The histopathological appearances suggest that many of the important pathological events occur early in the course of infection. By 12 to 16 h postinoculation, there is already the formation of relatively dense inflammatory aggregates and hyphal germination with evidence of early invasion (see Fig. 2). Hyphal extension that is initiated deep within areas of inflammation and necrosis invades into the contiguous lung, resulting in progressive pulmonary injury. The histopathological studies also indicate the various tissue compartments that are poten-tially important for clinicians treating invasive pulmonary aspergillosis. Immediately after the inhalation of conidia, epithelial lining fluid (ELF) along with structures comprising the alveolar-capillary barrier and the phagolysosome of pulmonary alveolar macrophages are likely to be important (10). Achieving therapeutic antifungal concentrations within the ELF and pulmonary alveolar macrophages is likely to be especially important at this early stage of infection. At later time points, hyphae are sequestered within areas of dense inflammation. At this stage, antifungal concentrations in lung parenchyma and blood may be more relevant than those in ELF. The sequestration of fungi within these inflammatory foci may represent an important obstacle for the timely attainment of effective antifungal concentrations.

Our study suggests that the (1→3)-β-D-glucan and galactomannan are inextricably tied to the pathogenesis of invasive pulmonary aspergillosis; this is consistent with our previous studies of galactomannan (12). The kinetics of both cell wall molecules appear comparable and provide similar information regarding the extent and stage of infection. Concentrations of both biomarkers begin to rise within the first 24 h of infection, well before pulmonary infarction is evident, supporting the concept that these molecules, when circulating, signal invasive disease almost immediately after host defenses are breached. The rapidly progressive histopathological changes that we observed suggest that the profiles of the biomarkers reflect progressive fungal infection rather than translocation of these molecules across the gut as a result of mucositis. Nevertheless, the latter may occur, at least to some extent. A further investigation of this possibility would require the measurement of galactomannan and (1→3)-β-D-glucan in uninfected immuno-suppressed rabbits, which was not performed in these studies.

The kinetic profiles of galactomannan in bronchoalveolar fluid and serum are completely distinct, suggesting that this large-molecular-weight antigen does not traverse the alveolar-capillary barrier. Using an in vitro model of the human alveolus, we have previously shown that galactomannan concentrations in the bloodstream begin to rise at the time hyphae penetrate into the pulmonary capillary (12). Although this is also likely in laboratory animals and humans, it is more difficult to provide irrefutable evidence. Using the same in vitro model, we have also shown that the antifungal exposure response
relationships are different in the alveolar and endothelial compartments, providing further evidence that these compartments are distinct from a pharmacological perspective. The response of galactomannan concentrations in serum and BAL to antifungal therapy requires further study. The trajectories of the biomarkers may be influenced, at least to some extent, by the species of laboratory animal and the experimental conditions used in each model. The trajectory of the biomarkers in this rabbit model following endobronchial inoculation of conidia may be somewhat different from other models of invasive pulmonary aspergillosis that use alternative modes of immunosuppression and inoculation (see, for example, reference 22).

Both molecules exhibit significant interindividual variability in the trajectories of their concentration-time profiles in rabbits. This is best appreciated using the Monte Carlo simulations. Some rabbits have relatively flat trajectories, whereas other rabbits have rapidly increasing concentrations. Importantly, the behavior of both biomarkers is stochastic (i.e., the trajectory of the biomarker is not predictable a priori). This variability is likely to be intrinsic to the pathogenesis of invasive pulmonary aspergillosis and is unavoidable despite the rigorous conditions under which these experiments were conducted. These observations are probably relevant for humans in whom the trajectory of fungal cell wall molecules is likely to be affected by various factors, such as the total fungal burden and the patient’s immunological status. The precise relationship between the behavior of the biomarker (quantified in terms of the trajectory or the biomarker AUC) and outcomes of clinical interest (e.g., survival) is relatively poorly understood. Furthermore, there may be significant strain-to-strain differences in the release of these biomarkers in the lung of laboratory animals and patients, and this requires further study.

Both clinical and preclinical data increasingly attest to the importance of early antimicrobial therapy for achieving optimal clinical outcomes (4, 5, 8, 11, 15, 16). This is reflected in the use of a variety of therapeutic strategies such as antifungal prophylaxis, empirical antifungal therapy, and preemptive therapy. For invasive pulmonary aspergillosis, early antifungal therapy may be beneficial for the following reasons. (i) Conidia and germings may be localized to ELF and to pulmonary alveolar macrophages where antifungal concentrations are attainable by triazoles, echinocandins, and polyenes (2, 9, 21). (ii) The antifungal activity of pulmonary alveolar macrophages against *Aspergillus* spp. occurs at an early stage principally against conidia through phagocytosis and nonoxidative mechanisms. This activity of pulmonary alveolar macrophages may be further enhanced through the combined interaction with antifungal agents (20). (iii) Sequestration of the organism within dense inflammatory infiltrates or in areas of hemorrhagic infarction may lead to suboptimal concentrations of

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**FIG. 8.** Effect of progressively delayed administration of amphotericin B deoxycholate (1 mg/kg) on fungal burden (A), infarct score (B), lung weight (C), and survival (D). ns, Not significant; *, *P* < 0.001. The data are means ± the SEM.
drug. (iv) Fungal growth rate may slow with increasing biomass or the generation of specific immunological responses (1). (v) Finally, organisms that grow more slowly tend to be less susceptible to antimicrobial agents (13). Our findings should be confirmed with other clinically relevant antifungal agents.

The clinical implications of our study are clear. Both (1→3)-β-D-glucan and galactomannan can detect infection almost immediately after the host is breached, but this requires relatively intense sampling. Sampling every 3 to 4 days (as is widely advocated) may mean that a diagnosis is made too late. Relatively small delays in the initiation of specific antifungal therapy appear to be detrimental. Clearly, therefore, the challenge is the identification of infection in an accurate, noninvasive, timely, and cost-effective manner. A diagnosis of invasive pulmonary aspergillosis must be followed by the prompt initiation of antifungal therapy.

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