Measuring intraspecific variation in the response of clinical isolates of Aspergillus fumigatus to oxidative stress.

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Research note

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Abstract

Objective In this study, the survival of clinical isolates of the pathogenic fungus Aspergillus fumigatus against the oxidative stressors, hydrogen peroxide and menadione, and UV light, was examined to see if there was variation between isolates and if the variation was linked to virulence. Results Fifteen isolates were tested, five from cases of invasive aspergillosis (IA isolates) and ten from cases where the fungus had colonised a patient (colonising isolates). Exposure to UV light and hydrogen peroxide did not show significant differences between the groups of isolates. Colonising isolates showed a trend for greater survival when treated with hydrogen peroxide, mean survival 18.9%, compared to IA isolates, mean survival 8%. Treatment with 50mM menadione confirmed this trend in colonising isolates with an average conidial survival of 72% compared to 50% in IA isolates. Overall significant sensitivity to 50mM menadione was observed in 1/10 colonising isolates compared to 4/5 IA isolates. Increased sensitivity to oxidative stress in IA isolates may seem counterintuitive but could be utilised as an indicator of pathogenic potential in isolates of the fungus and be used in further studies to unravel the complex interplay between host and pathogen.

Introduction

The ascomycete fungus *Aspergillus fumigatus* is a globally distributed decomposer of organic matter in the environment. Its broad distribution is due to its production of vast numbers of conidia which are easily distributed by wind currents. These conidia are inhaled by birds and mammals in which it can cause several disease states (1). In humans the most severe disease state is invasive aspergillosis, which typically affects immunocompromised individuals particularly those that are neutropenic (2). For conidia to enter the host and lead to successful infection of a human they must overcome a number of challenges including; UV radiation during dispersal (3), innate immune effectors such as antimicrobial peptides and phagocytosis by immune cells including alveolar macrophages (4), localised hypoxic conditions (5), and essential nutrient acquisition (6).

Conidia and early developmental stages of the fungus also face a particular challenge with regard to oxidative stress. Exposure to UV light can induce the production of reactive oxygen species within cells (7), countered in conidia by their high melanin content where melanin also plays a role in overcoming host defences, by resisting the oxidative burst produced by immune cells and in modulating the internal environment within the phagolysosomes of alveolar macrophages (8).

Resistance to innate host defences has been proposed to be a significant factor in the establishment of IA. Like bacterial pathogens it is increasingly clear that not all isolates of a pathogenic fungi are equally virulent (9, 10). Studies have described differences between environmental and clinical isolates of *A. fumigatus* with respect to virulence in murine models through quantification of pulmonary fungal burden and measurement of mortality (11, 12). In this study we compared the response of clinical isolates of *A. fumigatus* from patients that had IA (termed “IA isolates”), and those that did not develop IA but were colonised by the fungus, (termed “colonising isolates”), to oxidative stress by measuring conidial survival

in response to H_2O_2, menadione, and UV light. This may help to elucidate factors that may be relevant to the outcome of the host fungus interaction.

Methods And Materials

Organisms and strains: Fifteen strains of *A. fumigatus* were studied, and were obtained from the Centre of Infectious Diseases and Microbiology Laboratory Services, Westmead hospital. Ten (Af1 – Af10) were from patients colonised with *A. fumigatus* (colonising isolates) and five (Af11 -Af15), were from cases of proven IA (IA isolates). Cultures were grown on potato dextrose agar (PDA) for three days at 37C and conidia were isolated from each strain as previously described (13).

Measurement of response to hydrogen peroxide and menadione: To measure the effects of acute exposure to menadione (10mM stock in ethanol) and H_2O_2 (30% solution) *A. fumigatus* spore suspensions from each isolate, $1x10^6$ conidia/ml, were incubated for 3 hours at 37°C with 0 mM, 30 mM or 50 mM of menadione or H_2O_2. After incubation conidia were dilution plated onto PDA and incubated at 37°C for 24 hours and counted to determine CFU/mL. The percent inhibition was calculated relative to the 0 mM control.

Measurement of response to UV light: For each isolate, approximately 200 conidia were spread plated onto malt extract agar (MEA). Five plates inoculated with the same isolate were placed at different positions within a TopSafe PC2 Biosafety cabinet and UV irradiated (1.6 W/m2) for 1 minute. This was repeated for all 15 isolates. Following irradiation of each isolate, the biosafety cabinet was vented for 5 minutes to prevent ROS accumulation. Colony forming units (CFU) on control plates were counted following incubation at 37°C for 24h. An additional incubation for 24 hrs at 25°C preceded CFU counting of UV-irradiated plates. Percent survival for each isolate was calculated relative to a non-irradiated control and based on the average CFU counts across the five irradiated plates. The experiment was repeated four times, with the order in which isolates were irradiated changed to achieve uniform average UV-order position amongst all isolates.

Statistical Analysis: All data were analysed using Graphpad version 7, UV data was analysed using ANOVA and post-test (Dunn's) and menadione/H_2O_2 data were analysed by t-test. There were five replicates of each experiment.

Results And Discussion

The effect of hydrogen peroxide and menadione on clinical isolates of A. fumigatus*.* The addition of 30 mM H_2O_2 had a similar effect on all isolates tested, the average survival for colonising isolates was 58% and survival for IA isolates was 59%. Increasing H_2O_2 to 50 mM had a greater effect on survival and revealed greater differences between isolates, the average survival for colonising isolates was 18.9% and survival for IA isolates 8%. These broad average values suggest a difference between the two groups of isolates but the greater value for coloniser was caused by higher survival rates in just four isolates Af01 –

Af04. By comparing the results at 30mM and 50mM only one isolate, showed statistically significant resistance to H_2O_2 (Table 1).

The data for menadione indicate that it has a smaller effect on survival with increasing doses, this created the subtlety necessary to discriminate between isolates in terms of sensitivity or resistance to oxidative stress (Table 1). Addition of 30 mM menadione led to an average survival of 80% for colonising isolates and 76% for IA isolates. Addition of 50 mM led to an average survival of 72% in colonising isolates and 50% in IA isolates. This is a more pronounced difference than for H_2O_2, the effect was not confined to a small number of isolates skewing the data, comparison of survival for each isolate at 30 mM and 50 mM revealed that 90% of colonising isolates were relatively resistant to menadione whereas this was just 20% for IA isolates (Table 1). These data suggest that there is a difference in the ability of *A. fumigatus* isolated from patients where the fungus was just a coloniser compared to isolates that caused invasive disease with respect to tolerance of oxidative stress. However, this would require confirmation in larger scale studies.

The effect of acute exposure to UV on clinical isolates of A. fumigatus*.* There were only four isolates that were relatively resistant to UV light exposure and these showed >45% survival, the most sensitive isolates showed 15% survival (Figure 1). Resistance was not associated with whether colonising (three isolates) or IA isolates (one isolate) were tested. These data do not suggest that there are any important differences between isolates with regard to UV sensitivity, there was no clustering of sensitive or resistant isolates based on origin of the isolate. UV resistance in conidia is related to the melanin in the conidial cell wall which mitigate UV damage to ensure spore survival (8). However, modulating UV intensity was not possible in our experimental setting and therefore we could not modify the experiment to detect suitably subtle variations in response of isolates to UV. An element of UV toxicity is related to the generation of oxidative stress in host cells; isolates Af01, Af04, Af09, and Af14 that showed relative resistance to UV were also resistant to treatment with 50 mM menadione. However, only Af01 and Af04 showed resistance to H_2O_2 exposure. These suggest an overlap in resistance to UV damage and menadione. Studies in *Saccharomyces cerevisiae* indicated that different genes were induced during exposure to H_2O_2 compared to menadione (14) so it should be expected that isolates might display different survival characteristic when comparing both stressors.

Phenotypic variation and virulence. There have been studies that have compared the phenotypic characteristics of clinical and environmental isolates of *A. fumigatus*. It has been observed that the rate of radial growth on agar plates is correlated with virulence in *A. fumigatus*, with faster growing isolates being more virulent (15). Further, it has been found that cell wall chitin content and hyphal diameter in an environmental isolate led to decreased virulence in this isolate showing further characteristics that could be considered important in characterising virulent isolates (16). The key characteristic that has been used to compare isolates has been virulence in murine models of IA. In a study using mixed isolate infections it was found that the more virulent isolates had a clinical origin but the degree of virulence in the environmental isolates suggested that they would still have the capacity to cause infection (11). Another study tested the hypothesis that certain isolates of *A. fumigatus* are more virulent than others and was

able to demonstrate this in a murine model, finding that environmental isolates were less virulent than clinical isolates (12). These studies support the findings that intraspecific variation in virulence exists in *A. fumigatus* but the mechanisms have been difficult to identify. This is a particular problem with an opportunistic pathogen, which does not possess a specific virulence mechanism.

The host response to *A. fumigatus* has been studied as a means to define pathogenicity by interacting the isolates of the fungus with monocyte-derived dendritic cells and mice (17). The study found that mo-DCs produced strain specific cytokine responses; it was further observed that different wild-type isolates of the fungus induced inflammatory or hyperinflammatory responses in immunocompetent mice and that these differences in inflammatory response had a strong effect on the outcome of infection (17). It may seem counterintuitive that the IA isolates showed reduced resistance to oxidative stress induced by menadione compared to colonising isolates but if host immune interactions determine the outcome of IA then factors linked to this reduced oxidative resistance may be crucial in the development of IA making these isolates of value in future host-interaction studies.

Limitations

Limitations of this research focus on the need to test a greater number of isolates from more clinical centres to have a true representation of the oxidative stress sensitivity in the species. Ideally, we could have used a greater variety of doses of H_2O_2 to find a concentration that could show variation between isolates. A further limitation is that it would have been ideal to have characterised the virulence of each strain of the fungus.

List Of Abbreviations

H_2O_2, hydrogen peroxide. IA, invasive aspergillosis. UV, ultraviolet. PDA, potato dextrose agar. CFU, colony forming unit.

Declarations

Author’s Contributions:

SEM and CR performed the experimental work for the study. AP assisted in experimental design and preparation of the manuscript. CH and SC assisted in experimental design and preparation of the manuscript and provided the clinical isolates. COM designed the study, assisted in data analysis and wrote the manuscript.

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Competing interests:

The authors declare that they have no competing interests that could affect the integrity of this study.

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The data supporting the results of this study are included within this article; raw data can be requested from the corresponding author.

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Tables

Table 1 Effect of acute exposure (3 h) to menadione and H_2O_2 on the survival of conidia of clinical strains of *A. fumigatus*.

Isolate	Menadione					H_2O_2				
	30 mM		50 mM			30 mM		50 mM		
	Mean[a]	SD	Mean	SD	p-value[b]	Mean	SD	Mean	SD	p-value
Af01	70.5	25.5	65.6	13.0	**0.71**	67.5	17.0	44.8	8.6	0.03
Af02	83.0	8.8	71.0	9.3	**0.07**	63.5	16.7	29.5	6.8	0.003
Af03	87.4	5.7	77.2	6.0	0.025	65.5	14.2	25.2	6.5	0.0004
Af04	79.7	13.4	72.2	12.7	**0.39**	63.6	11.8	40.1	19.8	**0.06**
Af05	76.8	13.2	60.8	12.6	**0.086**	73.8	5.6	18.9	4.2	0.0001
Af06	70.8	14.9	64.8	9.1	**0.46**	65.8	14.0	11.2	4.0	0.0001
Af07	76.4	12.7	68.1	12.8	**0.34**	43.9	7.6	6.5	2.4	0.0001
Af08	81.2	15.0	77.4	16.0	**0.703**	62.9	12.1	6.3	2.7	0.0001
Af09	87.6	3.9	79.6	3.5	**0.0092**	38.5	13.4	3.8	1.6	0.0004
Af10	88.3	8.2	78.3	5.8	**0.06**	37.3	8.1	3.5	1.0	0.0001
Af11	69.2	17.8	43.0	16.3	0.0414	46.7	16.1	0.6	0.6	0.0002
Af12	81.5	11.8	47.8	10.4	0.0014	70.3	18.9	4.1	3.6	0.0001
Af13	77.2	7.2	48.4	15.9	0.0062	48.0	16.8	0.7	0.8	0.0002
Af14	73.2	33.2	59.2	17.4	**0.43**	71.2	9.5	5.5	3.3	0.0001
Af15	77.1	7.4	53.9	8.5	0.0017	59.9	15.4	29.2	4.9	0.003

[a] Mean of treatment relative to untreated control from five replicate experiments. [b] p-Value from t-test of 30 mM data compared to 50 mM data. Values in bold indicate isolates that showed no significant changes in inhibition between treatments with 30 mM and 50mM of the oxidative stressor.

Figures

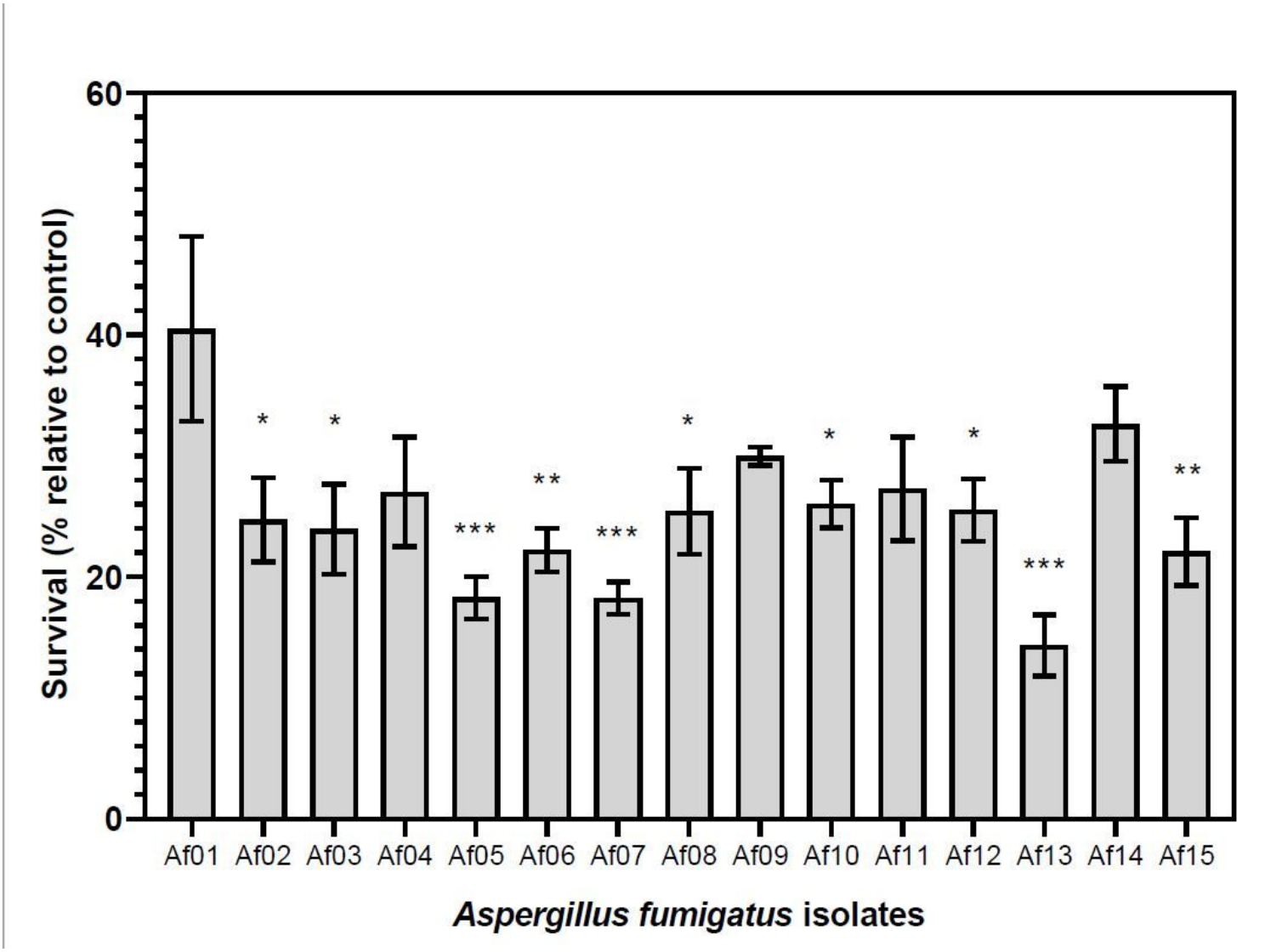

Figure 1

Induction of oxidative damage in 15 isolates of A. fumigatus through exposure to UV light (1.6 W/m2) for one minute. Data is expressed as percentage of CFUs surviving compared to untreated controls. Data represents mean and standard errors from five replicate experiments for each isolate. Data was analysed by one-way ANOVA with multiple comparison of all isolate means.