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Comparison of extracellular phospholipase activities in clinical and environmental *Aspergillus fumigatus* isolates

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Extracellular phospholipase production by environmental and clinical isolates of *Aspergillus fumigatus* collected from several centres world-wide were compared. All isolates produced extracellular phospholipases which included phospholipase C and a phospholipid acyl hydrolase (phospholipase A and/or phospholipase B) activity. Clinical isolates of *A. fumigatus* produced the largest zone sizes in a diffusion assay and clinical isolates produced more extracellular phospholipase C than environmental isolates. However, environmental isolates of *A. fumigatus* showed increased acyl hydrolase activity compared to clinical isolates of *A. fumigatus*. This study suggests that extracellular phospholipase C activity, but not extracellular acyl hydrolase activity may be important in the pathogenicity of *A. fumigatus*.

Introduction

Over the last two decades there has been a substantial increase in the incidence of opportunistic infectious disease caused by members of the genus *Aspergillus* [1–4]. Four species, *A. fumigatus, A. flavus, A. terreus* and *A. niger* account for the vast majority of all infections, with *A. fumigatus* accounting for around 90% of these [3]. It is widely accepted that the pathogenic aspergilli are likely to possess attributes that enable them to cause disease in man and animals such as the ability to grow at 37°C and small spore size allowing penetration into the alveoli [3,4]. Several factors have been proposed as putative virulence determinants in *A. fumigatus* [5]. The ability of *A. fumigatus* conidia to bind several matrix proteins, including laminin and fibrinogen has been well documented [6–10] and may play a role in the establishment of infection although the specific adhesin molecules have yet to be isolated.

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A. fumigatus is known to produce a wide variety of mycotoxins including gliotoxin and restrictocin [11–16]. The importance of these and other toxins is unclear with respect to pathogenesis. Although both can be detected during animal and human infection at levels known to cause cellular damage *in vitro* [11,17–19], non-gliotoxin producing species of *Aspergillus* are pathogenic and restrictocin null mutants cause similar levels of mortality to wild-type strains in animal models [20–22].

The production of extracellular proteases has been proposed by several authors as potential pathogenicity determinants and a number of different endopeptidases (including alkaline protease, metalloprotease and aspartic protease) have been identified [4,23–25]. Mutants lacking the ability to produce one or more of these proteases have little impact on mortality rates in animal models [22,26–29] although a role in allergy is possible [30,31].

The production of extracellular phospholipases have been shown to be important virulence determinants in the pathogenesis of several bacterial infections including those caused by *Clostridium perfringens, Pseudomonas aeruginosa* and *Listeria monocytogenes* [32,33]. Phospholipase B production by *Candida albicans* and *Cryptococcus neoformans* have been correlated with virulence [34–37] and phospholipase B knockout

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mutants in *C. albicans* and *C. neoformans* shown to have reduced pathogenicity in animal models [38,39].

Previously, using FAB–MS (fast atom bombardment–mass spectroscopy) analysis of phospholipid breakdown products from *A. fumigatus* culture broths, we identified ions consistent with the secretion of several phospholipase classes, including phospholipase B and phospholipase C [40]. This contrasts with *C. albicans* and *C. neoformans* which only appear to secrete phospholipase B with additional lysophatidylphopholipase and transacylase activities [41–43].

In this study we compared the extracellular phospholipase activities of clinical and environmental isolates of *A. fumigatus* and found significantly higher levels of extracellular phospholipase C activity in clinical *A. fumigatus* isolates.

Materials and methods

Storage and cultivation of isolates

Clinical and environmental isolates of *A. fumigatus* were obtained from the *Aspergillus* culture collection based at Hope Hospital, Salford, UK. These strains include isolates from a number of centres from the UK, USA and Europe.

Isolates were stored as conidial suspensions in 20% (v/v) glycerol at −80°C until needed. Conidia for inoculation of lipid media were obtained from cultures grown on Sabouraud Dextrose agar at 37°C for 48–96 h by gentle agitation in PBS containing 0.01% (v/v) Tween 80 and filtered through two layers of lens tissue.

Conidial suspensions were counted using a haemocytometer and the concentration adjusted to 1×10^7 conidia per ml. 1 ml of the conidial suspension was inoculated into 250-ml Erlenmeyer flasks containing 49 ml filter sterilized (0.22 μm) 0.5% (w/v) soy bean phospholipid supplemented with 1 ml Vogel salts [44]. Flasks were incubated at 37°C with constant shaking (200 r.p.m.) for 55 h and filtered through four layers of sterile muslin. Supernatants were filter sterilized prior to freezing at −80°C.

Phospholipase assays

Culture supernatants were removed from storage at −80°C and thawed on ice prior to use in assays. Estimates of total phospholipase activity were made using an agar diffusion assay based on the cup plate assay of Tseng and Bateman [45]. Wells (8 mm diameter) were cut into 20-ml diffusion plates (0.5% (w/v) soy bean phospholipid, 50 mmol/l $CaCl_2$, 1% (w/v) agar) using a sterile cork borer and 150 μl filter-sterilized culture supernatant added. Plates were incubated at 37°C for 4 days and the diameters of any zone of precipitation measured.

Culture supernatants were assayed for extracellular phospholipase C, using the synthetic substrate p-nitrophenyl phosphorylcholine, by monitoring the liberation of nitrophenol as previously described [40]. Phospholipase A and B (phospholipid acyl hydrolase activity) were measured using the synthetic substrate 4-nitro-3-octanoyloxy benzoic acid (NOBA) according to the method of Cho and Kezdy [46]. This method was originally used to determine phospholipase A_2 activity; however, we have recently shown that it also acts as a substrate for phospholipase B (unpublished observations). Therefore, this assay can be used to measure total phospholipid acyl hydrolase activity (i.e. a combination of both phospholipase A and B). Briefly NOBA was added to assay buffer (10 mm Tris, 10 mmol/l $CaCl_2$, 0.1 mol/l NaCl, pH 8.0) to a concentration of 1 mg/ml. Supernatant was mixed with the substrate in a ratio of 1:60 before incubation at 37°C. The absorbance of the reaction mixture was monitored periodically over time at 410 nm and the rate of formation of the chromogenic reaction product 3-hydroxy-4-nitrobenzoic acid determined. All strains were analysed in duplicate with triplicate readings made for each assay.

The protein concentration of the culture supernatants were estimated using the BCA protein assay kit (Pierce, UK).

Statistical analysis of the data was performed using the SPSS computer program using least significant difference analysis. Correlations were performed using Pearson's coefficient of linear correlation.

Results

A total of 53 clinical and 11 environmental isolates of *A. fumigatus* were tested for growth and production of extracellular phospholipases on lipid medium (Table 1). All isolates were able to grow to some degree in liquid media with soy phospholipid as the sole carbon source; however, environmental *A. fumigatus* isolates produced significantly less biomass ($P < 0.05$) compared to clinical isolates over the incubation period (Table 1). In addition, four of the 11 environmental *A. fumigatus* isolates were unable to clear the turbid phospholipid media indicating residual phospholipid. Repeated subculturing (four times) for two of these isolates did not change the level of secreted phospholipases (results not shown). The total zone sizes in the agar diffusion assay ranged from 15.1 to 35.1 mm with each zone consisting of a number of inner zones with differing intensities of

Table 1 Mean values and ranges for zone of precipitation, phospholipase C activity, phospholipid acyl hydrolase activity and dry weight for clinical and environmental isolates of *Aspergillus fumigatus* grown for 55 h in 0.5% (w/v) egg yolk lecithin supplemented with Vogel's mineral salts. n = number of isolates in each category. Results represent the mean value of all isolates in each group ± SEM. One unit (U) is defined as the amount of phospholipase activity required to cleave 1 μmol substrate per hour at 37°C, pH 7.4. The mean values for the environmental and clinical isolates were significantly different in all cases ($P < 0.05$).

	n	Phospholipase C activity (U/mg protein)		Phospholipid acyl hydrolase activity (U/mg protein)		Zone Diameter mm		Dry weight (mg/ml)
		Mean (SE)	Range	Mean (SE)	Range	Mean (SE)	Range	Mean ± SE
A. fumigatus clinical	53	1.39 ± 0.15	0.11–5.38	4.09 ± 0.45	0.41–9.34	25.01 ± 2.7	15.0–35.1	3.44 ± 0.05
A. fumigatus environmental	11	0.55 ± 0.15	0.12–1.53	5.90 ± 0.68	3.24–8.45	18.3 ± 1.6	16.6–19.6	2.52 ± 0.05

precipitation (results not shown). Clinical *A. fumigatus* isolates were found to produce significantly ($P < 0.05$) larger zone sizes (mean 25.01 ± 2.70 mm) than that observed for the environmental isolates (mean 18.30 ± 1.60 mm).

Clinical isolates of *A. fumigatus* as a group had an extracellular phospholipase C activity that was ca. 2.5-fold higher than the mean activity from environmental isolates (mean 1.39 ± 0.15 U/mg protein and 0.55 ± 0.15 U/mg respectively). Acyl hydrolase activity was also detected in all isolates tested but was around 1.4-fold higher in the environmental isolates as a group compared to the clinical *A. fumigatus* group (mean 5.90 ± 0.68 U/mg protein and 4.09 ± 0.45 U/mg protein respectively).

A positive correlation was seen between phospholipase C activity and total zone size for all isolates of *A.fumigatus* ($r = 0.67$) but no correlation was found between total acyl hydrolase activity and total zone size (Fig. 1) or between phospholipase C and acyl hydrolase activities (data not shown). When correlations were calculated within each group between zone size and phospholipase C activity, environmental isolates of *A. fumigatus* had the lowest correlation value ($r = 0.61$) compared to clinical *A. fumigatus* isolates ($r = 0.82$).

Discussion

In this study, we compared total phospholipase, phospholipase C and phospholipid acyl hydrolase activities between clinical and environmental isolates of *A. fumigatus.* Clinical *A. fumigatus* isolates were seen to produce larger zones sizes compared to the environmental isolates as determined by the diffusion agar assay [45]. Previously this method has been used to demonstrate a strong positive correlation between zone size and the level of extracellular phospholipase activity and has shown that clinical isolates of *C. albicans* and *C. neoformans* produce higher levels of extracellular phospholipase activity compared to environmental isolates [34–37]. Because all the extracellular phospholipases produced by an individual isolate would be present in the culture supernatant, the total zone size was used as an indication of the total phospholipase activity of an isolate. Interestingly, a positive correlation was found between the level of measurable extracellular phospholipase C activity in the culture broths and the zone size, with this correlation being strongest in the clinical isolates which are the highest phospholipase C producers, and lowest in the environmental isolates which are the weakest producers. However, there was no correlation between extracellular acyl hydrolase activity and zone size, suggesting that much of the observed precipitation was due to the action of extracellular phospholipase C (Fig. 2).

In *C. neoformans* and *C. albicans*, extracellular phospholipase B (lysophospholipase) appears to be

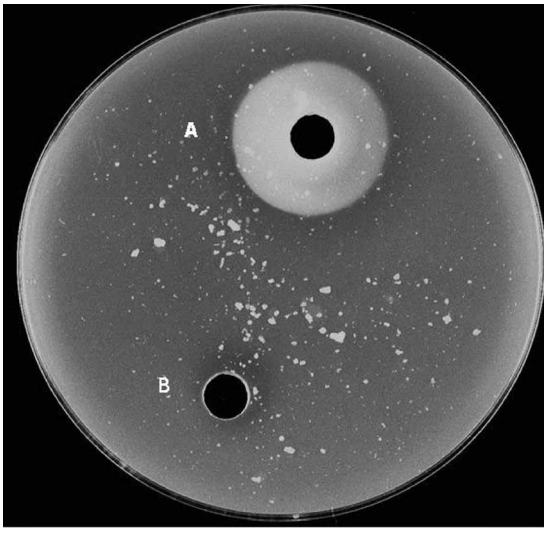

Fig. 1 Diffusion plate assay using filter sterilised culture supernatant from *Aspergillus fumigatus* isolate AF10 grown for 55 h in Vogel's medium with either (A) 0.5% (w/v) egg yolk lecithin or (B) 0.5% (w/v) glucose as sole carbon source. 150 μl of filter sterilised supernatant was added to the respective wells and incubated at 37°C for 4 days.

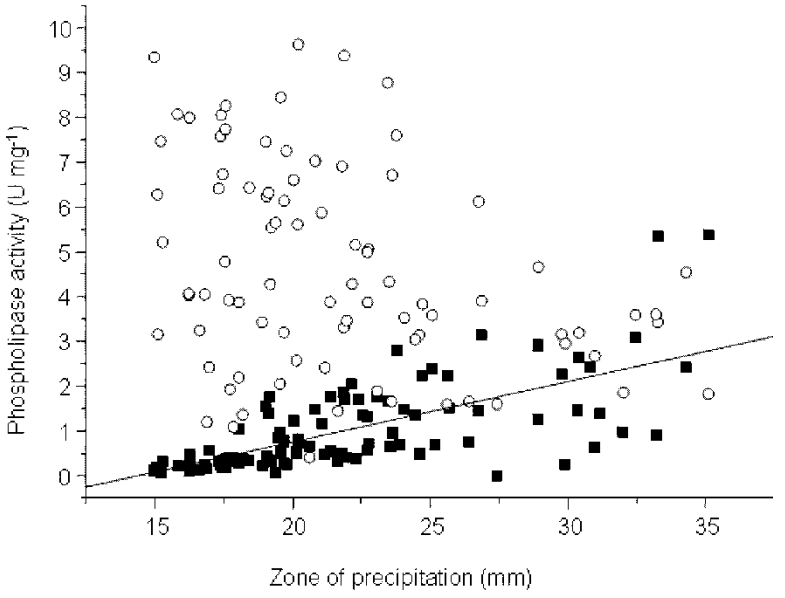

Fig. 2 Correlation between total zone diameter and extracellular phospholipase C activity (○) or extracellular phospholipid acyl hydrolase activity (■) for each individual isolate used in this study. A positive correlation existed between zone size and phospholipase C activity ($r = 0.67$) but not between total zone size and phospholipid acyl hydrolase activity ($r = 0.33$).

the only secreted phospholipase [41–43] and an attenuation of pathogenicity has been demonstrated in mice in knockout strains [38,39]. *A. fumigatus* contains at least two phospholipase B genes with high homology to other fungal phospholipase B/lysophospholipase B genes (NCBI Accession No. AF223004 and AF223005). A blastx interrogation of the *A. fumigatus* genome sequence (TIGR database) with a highly conserved region from other eukaryotic phospholipase A proteins did not reveal any homologues. Although a putative phospholipase A gene has been reported from *A. oryzae* [47], a blastx search of this sequence against the NCBI database gave highest matches against fungal triacylglycerol lipases and did not contain eukaryotic phospholipase conserved regions (data not shown). Thus it seems likely that the extracellular acyl hydrolase activity measured in culture supernatants of *A. fumigatus* represents phospholipase B activity alone. In this study however, phospholipid acyl hydrolase activity was significantly lower in the clinical isolates of *A. fumigatus* compared to the environmental isolates, exactly the opposite of what had previously been observed in similar comparative studies in *C. albicans* and *C. neoformans* [34–37]. This suggests that phospholipid acyl hydrolase activity for the environmental isolates may be more important for growth in the environment than it is for clinical isolates growing in the body.

Previously, we demonstrated the production of extracellular phospholipase C activity by *A. fumigatus* [40]. In this study comparing clinical and environmental isolates of *A. fumigatus*, although a wide variation in production was observed, clinical isolates produced significantly more phospholipase C than environmental isolates (Table 1). Extracellular phospholipase C activity was also correlated with total zone size indicating that phospholipase C activity makes a significant contribution to the total extracellular phospholipase activity. The importance of phospholipase C as pathogenicity determinant in the aspergilli is as yet unknown, but phospholipase C has long been known to be a highly significant pathogenicity determinant in several bacterial infections [32,33,48]. Moreover, aspergillosis is a disease almost exclusively acquired by inhalation of airborne conidia which penetrate deep into the alveolar spaces that are lined with lung surfactant which is composed of up to 80% phospholipid [49]. Degradation of lung surfactant and subsequent breakdown in oxygen tension may prove beneficial to colonisation. In addition, an increase in phospholipase activity has been demonstrated during the course of infection with the respiratory pathogen *Pneumocystis carinii* [50]. Genetic variants of surfactant may be more 'biodegradable' by phospholipase C or other phospholipases, accelerating the development of severe allergy, manifest as allergic bronchopulmonary aspergillosis [51].

Recently, we have identified and cloned a number of genes encoding extracellular phospholipases which includes three genes encoding putative secreted phospholipase C and differ significantly from the intracellular phosphatidylinositiol-specific phospholipases which are involved in intracellular signalling (unpublished data). Interrogation of the genome of the sequenced strain (AF293) reveals all three are present as a single copy and has been confirmed by Southern analysis in another clinical strain AF10 (unpublished data). Thus, differences in phospholipase C activity between environmental and clinical isolates are not due to differences in gene copy number but may be due to differences in regulation. Further investigations including studies on gene expression and the creation of null mutants are currently being undertaken.

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