CORE
Metadata, citation and similar papers at core.ac.uk
Provided by MURAL - Maynooth University Research Archive Library

Mycopathologia (2006) 161: 377–384
DOI 10.1007/s11046-006-0021-1

© Springer 2006

Susceptibility of larvae of *Galleria mellonella* to infection by *Aspergillus fumigatus* is dependent upon stage of conidial germination

Julie Renwick, Paul Daly, Emer P. Reeves & Kevin Kavanagh
Department of Biology, NUI Maynooth, Medical Mycology Unit, National Institute for Cellular Biotechnology, Co. Kildare, Ireland

Received 18 October 2005; accepted in revised form 2 February 2006

Abstract

The ability of conidia of the human pathogenic fungus *Aspergillus fumigatus* to kill larvae of the insect *Galleria mellonella* was investigated. Conidia at different stages of the germination process displayed variations in their virulence as measured using the *Galleria* infection model. Non-germinating ('resting') conidia were avirulent except when an inoculation density of 1×10^7 conidia per insect was used. Conidia that had been induced to commence the germination process by pre-culturing in growth medium for 3 h were capable of killing larvae at densities of 1×10^6 and 1×10^7 per insect. An inoculation density of 1×10^5 conidia per insect remained avirulent. Conidia in the outgrowth phase of germination (characterised as the formation of a germ tube) were the most virulent and were capable of killing 100% of larvae after 5 or 24 h when 1×10^7 or 1×10^6 conidia, that had been allowed to germinate for 24 h, were used. Examination of the response of insect haemocytes to conidia at different stages of the germination process established that haemocytes could engulf non-germinating conidia and those in the early stages of the germination process but that conidia, which had reached the outgrowth stages of germination were not phagocytosed. The results presented here indicate that haemocytes of *G. mellonella* are capable of phagocytosing *A. fumigatus* conidia less than 3.0 μm in diameter but that conidia greater than this are too large to be engulfed. The virulence of *A. fumigatus* in *G. mellonella* larvae can be ascertained within 60–90 h if infection densities of 1×10^6 or 1×10^7 activated conidia (pre-incubated for 2–3 h) per insect are employed.

Key words: *Aspergillus fumigatus*, *Galleria mellonella*, insect haemocytes, pathogenicity, virulence

Introduction

Insects are increasingly been viewed as a valid and useful model for evaluating the virulence of a range of human pathogens due to the structural and functional similarities between their immune system and the innate immune response of mammals [1, 2]. The innate immune system of mammals plays a critical role in defending the body from microbial pathogens and consequently data obtained with insects bear a strong similarity to the response seen in mammals [3, 4]. Larvae of the insect *Galleria mellonella* have been used to differentiate between pathogenic and non-pathogenic yeast species [5] and a positive correlation between the immune response of *G. mellonella* and mice to mutants of *Candida albicans* has been established [6]. Recent work has established the role of gliotoxin in the virulence of the human pathogen *Aspergillus fumigatus* to *Galleria* larvae [7] and factors affecting the virulence of *C. albicans* mutants in *Galleria* larvae have been elucidated [8]. The use of *Galleria* larvae for quantifying the virulence of mutants of *A. fumigatus* by introducing conidia into the haemocoel via the last left pro-leg has been demonstrated [9]. *G. mellonella* have also

been utilised as a model for studying the virulence of *Cryptococcus neoformans* [10]. Bhabhra et al. [11] have used an insect model to assess the virulence of mutants of *A. fumigatus* lacking the nucleolar protein *Cgr A*. In addition larvae of silkworms have been utilised to measure the efficacy of antibiotics in killing bacteria and a positive correlation with the results obtained from murine studies has been demonstrated [12].

Given the increasing use of insects as models for studying the virulence of fungal and bacterial pathogens and the strong ethical and economic imperatives to reduce the use of mammals in this form of testing it is essential that factors affecting microbial virulence are identified so that standardised inoculation protocols for use with insects can be developed. The aim of the work presented here was to ascertain whether the process of conidia germination affected the virulence of *A. fumigatus* in *Galleria* larvae and to establish whether this could be correlated with a reduction in the insect's ability to withstand the pathogen.

Materials and methods

Insect larvae

Sixth instar larvae of the wax moth *G. mellonella* were obtained from the Meal Worm Company (Sheffield, England). Prior to use larvae were stored in wood shavings in the dark at 15 °C and were used within 3 weeks of delivery.

Fungal strains and culture conditions

A. fumigatus ATCC 26933 (obtained from the American Type Culture Collection) was maintained on MEA agar (2% (w/v) malt extract (Oxoid Ltd., Hampshire, UK), 0.1% (w/v) peptone, 2% (w/v) glucose (Oxoid), and 2% (w/v) agar (Difco)) at 4 °C and sub-cultured every 3 months. *A. fumigatus* cultures were grown on MEA agar plates at 37 °C for 7–14 days and sporulating *A. fumigatus* colonies were washed with 0.1% (v/v) Tween-80 (Merck) surfactant solution to isolate conidia. Conidia suspensions were centrifuged (Beckman, GS-6) at 1500×*g* for 5 min, washed three times in phosphate buffered saline (PBS, pH 7.4) (Gibco) and resuspended in 5 ml PBS. Conidia density was determined using a haemocytometer after the suspension was diluted by a factor of 1:100 in PBS. A known concentration of conidia was placed in MEM culture medium (Sigma Aldrich) supplemented with 5% (v/v) foetal calf serum (FCS) (Gibco, Paisley, UK), 4 mm L-glutamine (Gibco) and incubated at 37 °C in an orbital shaker at 200 rpm for pre-determined periods of time to reach the different stages of conidial germination.

Inoculation of G. mellonella larvae

G. mellonella larvae, in groups of ten, were inoculated with conidia by injecting 20 μl of suspension into the haemocoel through the last pro-leg. The syringe used was a SGE 1-ml gas tight syringe (Scientific Pty. Ltd., Melbourne, Australia) with a diameter of 0.75 mm as described [13, 14].

Control treatments were also included to ensure that neither the injection procedure nor the incubation period were responsible for any mortality observed. These controls involved *G. mellonella* larvae injected with 20 μl of sterile MEM culture medium. *G. mellonella* larvae were placed in Petri-dishes and incubated in the dark at 30 °C (a temperature which was previously identified as being optimum for culturing larvae infected with fungal pathogens [5, 6]). Mortality rates were recorded for 4–5 days post-injection. Mortality was assessed based on lack of movement in response to stimulation and discolouration (melanisation) of the cuticle. All experiments were performed on three independent occasions and representative sets of data are presented.

Identification of stages of germination of A. fumigatus conidia

Conidia germination in MEM culture medium was recorded microscopically to record activation (conidia swelling), germination (formation of germ tube) and outgrowth (extension of germ tube). Conidia germination was also monitored by PAMAS particle counter (SVSS-C) analysis with analysis and size distribution (ASD) software. Conidia isolated in MEM culture medium after various treatments were diluted in PBS by a factor of 1:100 and conidia diameter was determined in a 1–8 μm range.

Phagocytosis by G. mellonella haemocytes of A. fumigatus conidia

Measurement of haemocyte-induced phagocytosis of conidia was based on the method of Arumugam et al. [15]. Conidia of *A. fumigatus* (1×10^7/ml) were suspended in 10 ml Minimal Essential Medium (MEM) + 5% (v/v) FCS and incubated at 37 °C and 200 rpm in an orbital incubator. Conidia were inspected microscopically to determine the stage of germination and then washed three times by centrifugation at $1500 \times g$ for 10 min prior to resuspending in PBS. Conidia were inoculated into *G. mellonella* larvae at a density of 1×10^6/insect. *Galleria* were incubated for 2 h at 30 °C and then bled into 12 ml insect physiological saline (IPS) [150 mm NaCl, 5 mm KCl, 0.1 M Tris–HCl, 10 mm EDTA and 30 mm sodium citrate, at pH 6.9]. Haemolymph was centrifuged at $1500 \times g$ for 5 min and resuspended in a further 6 ml IPS. Following another centrifugation step the pellet was suspended in PBS-glucose (5 mm) (PBSG). Phagocytosis was recorded by counting the number of haemocytes that contained conidia out of 200 haemocytes and this count was performed in triplicate for each sample. Haemocytes were counted and viability assessed using the trypan blue exclusion assay. The viability of extracted haemocytes was approximately 87%.

Results

Stages in the germination process of A. fumigatus conidia

Conidia of *A. fumigatus* incubated in MEM culture medium were examined for the morphological features of germination i.e. activation (spore swelling), germination (the first appearance of a germ tube) and outgrowth which was determined to have commenced once the germ tube had reached twice the diameter of the parent conidium as ascertained using both microscopic examination and a PAMAS particle counter with analysis and size distribution (ASD) software. Each phase of germination is represented in the photomicrograph of germinating conidia on an haemocytometer (Figure 1). *A. fumigatus* conidia were observed to be activated after 2 h incubation and this phase lasted for approximately 3 h (mean diameter 3–3.5 μm). After 4 h incubation, 100% of *A. fumigatus* conidia in MEM showed the characteristics of activation i.e. conidia swelling. After an incubation period of 7 h conidia began germ tube formation with 30%–40% of conidia in the budding phase and only 6% of conidia in outgrowth phase (Figure 2). After 11 h incubation almost 90% of conidia were in the early stages of the outgrowth phase and showed a mean diameter of 4.0 μm.

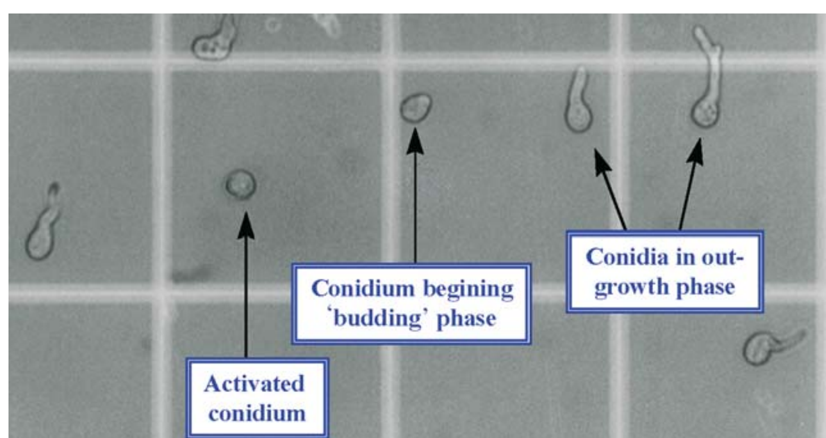


Figure 1. Morphological features of germination of *A. fumigatus* conidia based on activation (conidia swelling), germination (formation of germ tube) and outgrowth (germ tube formation). Conidia were viewed on a haemocytometer (Original magnification × 320).

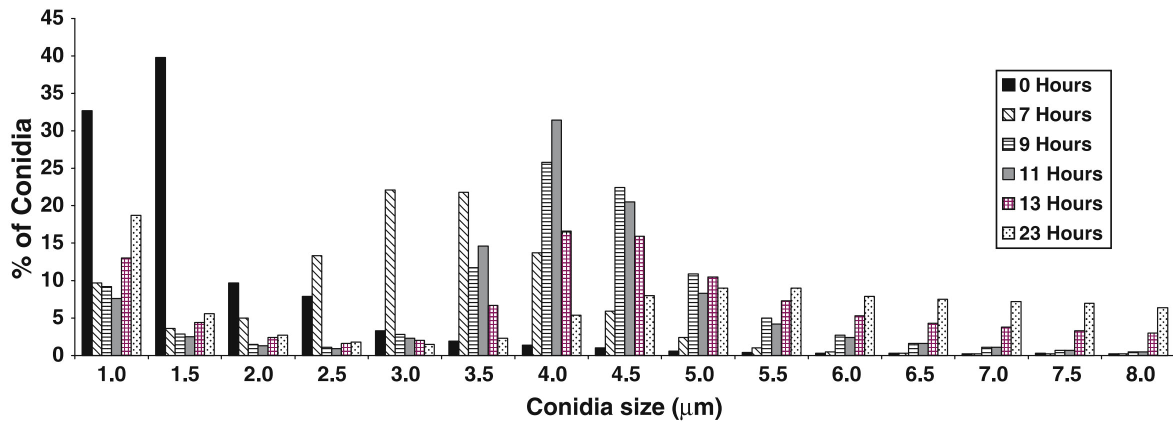


Figure 2. Size distribution of *A. fumigatus* conidia at different stages of the germination process. Conidia were induced to germinate by incubation in MEM culture medium at 37 °C for different periods of time (0, 7, 9, 11, 13 or 23 h). Conidia size was determined using a PAMAS particle counter equipped with size and distribution software.

Non-germinating conidia had a mean diameter of 1–1.5 μm but as germination commenced conidia were observed to swell and the mean diameter at 7 h had increased to 3–3.5 μm. At 9 h incubation the mean diameter of conidia was 4.0 μm. After 11 and 13 h incubation (times corresponding to the early outgrowth phase of germination) mean conidia diameter was 4–4.5 μm but at 23 h (when hyphal formation was observed) two peaks were apparent – one at 5.5 μm and a second peak at 1.0 μm which corresponded to the presence of hyphae in the culture.

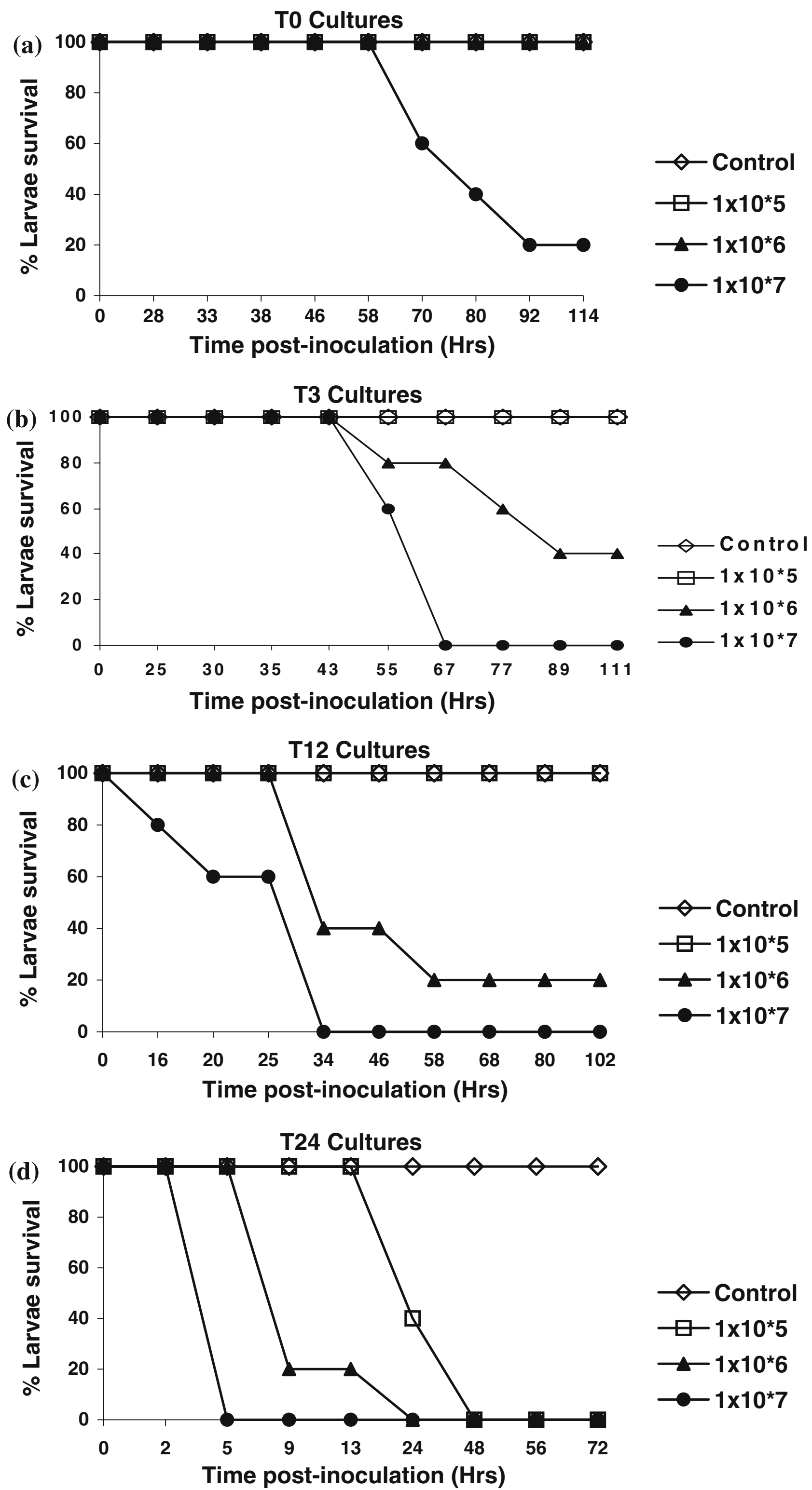
Effect of resting A. fumigatus conidia on the mortality levels of G. mellonella larvae

Non-germinating conidia at concentrations of $1 \times 10^5/20\ \mu l$, $1 \times 10^6/20\ \mu l$ and $1 \times 10^7/20\ \mu l$ in MEM culture medium were injected into *G. mellonella* larvae through the last pro-leg as described. Mortality rates were determined over a 114-h period. Resting conidia at time zero (conidia in MEM culture medium prior to incubation at 37 °C) varied in mean diameter from 1.0 to 1.5 μm (32.7% and 39.8%, respectively). Insects inoculated with conidia at concentrations of $1 \times 10^5/20\ \mu l$–$1 \times 10^6/20\ \mu l$ failed to cause larval death up to 114 h post-infection (Figure 3a). However, the highest concentration of resting conidia ($1 \times 10^7/20\ \mu l$) injected into *G. mellonella* larvae induced a mortality rate of 40% after 70 h and resulted in 20% survival after 92 h.

Effect of activated A. fumigatus conidia on the mortality levels of G. mellonella larvae

Activated *A. fumigatus* conidia (mean diameter 3 μm) introduced into *G. mellonella* larvae at a concentration of $1 \times 10^5/20\ \mu l$ failed to kill larvae even after an incubation period of 111 h. However, a conidia concentration of $1 \times 10^6/20\ \mu l$ caused 20% mortality after 55 h incubation, and 60% kill after 89 h incubation (Figure 3b). An increase in mortality was also recorded when *G. mellonella* larvae were injected with 1×10^7 conidia/20 μl. After 55 h 40% of larvae had died while after 67 h 100% had died. Therefore, activation of *A. fumigatus* conidia by incubation in MEM culture medium for up to 3 h and subsequent injection into *G. mellonella* larvae caused an increase in virulence when compared to non-activated conidia (Figure 3a) particularly when inoculum densities of 1×10^6 and 1×10^7/insect were used.

Figure 3. (a–d) Effect of *A. fumigatus* conidia on mortality of *G. mellonella* larvae. Larvae were inoculated with different densities of conidia that had been induced to germinate by pre-incubation in MEM culture medium at 37 °C for 0 h (a), 3 h (b), 12 h (c) or 24 h (d) to reach the activation stage (b), early (c) or late (d) outgrowth phases of germination. Larvae were inoculated with conidial densities of 1×10^5, 1×10^6 or 1×10^7 conidia/insect. The control consisted of larvae uninfected with conidia.


T0 Cultures
(a)
% Larvae survival
Time post-inoculation (Hrs)
Control
1x10*5
1x10*6
1x10*7
T3 Cultures
(b)
% Larvae survival
Time post-inoculation (Hrs)
Control
1x10*5
1x10*6
1x10*7
T12 Cultures
(c)
% Larvae survival
Time post-inoculation (Hrs)
Control
1x10*5
1x10*6
1x10*7
T24 Cultures
(d)
% Larvae survival
Time post-inoculation (Hrs)
Control
1x10*5
1x10*6
1x10*7

Effect of A. fumigatus conidia in outgrowth phase of germination on the mortality of G. mellonella larvae

Inoculation of *A. fumigatus* conidia, which had been incubated in MEM culture medium for 12 h to the early outgrowth phase, at a concentration of $1 \times 10^5/20\ \mu$l produced no larval death (Figure 3c). At a total conidial concentration of $1 \times 10^6/20\ \mu$l, *G. mellonella* larval mortality rates were 60%, 60% and 80% after 34, 46 and 58 h, respectively, post-inoculation. *G. mellonella* inoculated with conidia at a concentration of $1 \times 10^7/20\ \mu$l resulted in 100% mortality after 34 h.

Larvae of *G. mellonella* were inoculated with late outgrowth conidia at concentrations of 1×10^5, 1×10^6 and $1 \times 10^7/20\ \mu$l. A conidia inoculation of $1 \times 10^5/20\ \mu$l, resulted in 60% mortality after 24 h and 100% mortality after 48 h incubation. Infection with conidial concentrations of $1 \times 10^6/20\ \mu$l and $1 \times 10^7/20\ \mu$l resulted in 100% mortality at 24 and 5 h, respectively (Figure 3d).

Phagocytosis of A. fumigatus conidia by G. mellonella haemocytes

Larvae of *G. mellonella* were inoculated with conidia of *A. fumigatus* at different stages of the germination process (i.e. activation, germ tube formation and outgrowth) at a standard density (1×10^6/insect) and incubated for 2 h at 30 °C. The percentage of haemocytes that had attached or phagocytosed conidia was ascertained microscopically as described previously [15]. The results (Figure 4) indicate that using non-germinated (resting) conidia 62% of haemocytes had associated conidia. After 2 h incubation, when the conidia were in the early stages of the activation phase, the percentage of haemocytes showing associated conidia had decreased to 34%. Conidia allowed to germinated for 7 and 9 h to begin germ tube formation were less susceptible to haemocyte-mediated phagocytosis compared to conidia in the early stages of the activation process. The conidia that had been allowed to germinate for 11 and 13 h, to the early outgrowth stage, prior to inoculation were incapable of being phagocytosed by the haemocytes.

Discussion

Larvae of *G. mellonella* have been used previously to distinguish between *Aspergillus* species producing different levels of the immunosuppressive agent gliotoxin [7] and insects have also been used to quantify the role of *rel A* gene in contributing to the virulence of *P. aeruginosa* [16] and the virulence of mutants of *A. fumigatus* lacking the nucleolar protein Cgr A [11]. There is increasing attention being

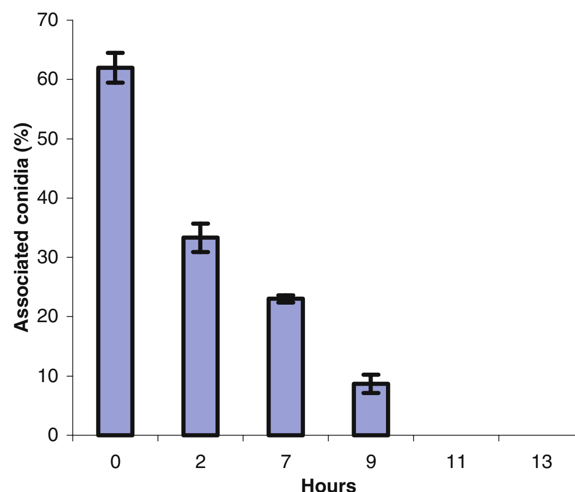


Figure 4. Interaction of *A. fumigatus* conidia at different stages of the germination process with insect haemocytes. Conidia were pre-incubated in MEM culture medium to reach the activation stage (2 h), germ tube formation stage (7 and 9 h) or outgrowth stage (11 and 13 h) of germination prior to infection of *G. mellonella* larvae. The percentage conidia associated with haemocytes refers to the number of haemocytes with bound or internalised conidia. Values are the mean ± SD of three independent determinations.

focused on the use of insects for measuring changes in microbial virulence – a process which has time and cost benefits to the researcher as well as ethical benefits in reducing the need to use mammals for this form of testing [3].

The results presented here demonstrate that the stage of conidial germination affects the virulence of *A. fumigatus* in *G. mellonella* larvae. Examination of the ability of *Galleria* haemocytes to engulf *Aspergillus* conidia revealed decreased phagocytosis as conidia increased in size from resting conidia (mean diameter (1–1.5 μm) to activated conidia (mean diameter 3–3.5 μm) through to those in the outgrowth phase (mean diameter 4–4.5 μm).

Haemocytes have been classified as prohaemocytes, plasmatocytes, granulocytes (granular cells), coagulocytes, spherulocytes and oenocytoids [17, 18]. The plasmatocytes and granulocytes participate in phagocytosis, nodule formation and encapsulation [19] which are important elements of the insect's cellular defence against bacteria and unicellular fungi [20]. Within *Drosophila melanogaster*, plasmatocytes participate, to some extent, in the synthesis of antimicrobial peptides during the humoral response [21, 22] and assume the function of phagocytosis of micro-organisms [23] while lamellocytes and crystal cells play respective roles in encapsulation and melanisation of larger intruders [24]. The decreased ability of haemocytes to engulf germinating conidia may be a result of their increased size but could also be due to the fungus secreting toxin(s) [7] or enzymes [25] which arrest the phagocytic process. Proteases produced by *Aspergillus flavus* have been implicated in the ability of this fungus to colonise *G. mellonella* [25].

The work presented here demonstrates that larvae of *G. mellonella* are susceptible to infection by conidia of *A. fumigatus* but that the stage of conidial germination and the infection densities are critical factors in producing larval death. Based on the size data for germinating conidia it appears that haemocytes are incapable of phagocytosing conidia with a mean diameter up to 3.5–4.5 μm. For *in vivo* assays to ascertain the relative virulence of *A. fumigatus* an inoculation density of 1×10^6 or 1×10^7/insect of activated conidia (pre-incubated for 3 h) would appear to be optimal in terms of giving results within 60–90 h and in providing conidia against which the insect immune system can mount a cellular response. Non-germinating conidia are avirulent at infection densities of 10^5 and 10^6/insect. Conidia in the outgrowth phase of growth appear to be too large for the haemocytes to engulf and may thus prevent an examination of the relative virulence of different *A. fumigatus* isolates.

Acknowledgements

This work was supported by funding from the Higher Education Authority of Ireland through the Programme for Research in Third Level Institutions cycle 3.

References

1. Ratcliffe N. Invertebrate immunity – a primer for the non-specialist. Immunol Lett 1985; 10: 253–270.
2. Salzet M. Vertebrate innate immunity resembles a mosaic of invertebrate immune responses. Trends Immunol 2001; 22: 285–288.
3. Kavanagh K, Reeves EP. Exploiting the potential of Insects for *in vivo* pathogenicity testing of Microbial pathogens. FEMS Microbiol Rev 2004; 28: 101–112.
4. Bergin D, Reeves EP, Renwick J, Wientjes FB, Kavanagh K. Superoxide production in haemocytes of *Galleria mellonella* – identification of proteins homologous to the NADPH oxidase complex of human neutrophils. Infect Immun 2005; 73: 4161–4173.
5. Cotter G, Doyle S, Kavanagh K. Development of an insect model for the *in vivo* pathogenicity testing of yeasts. FEMS Immunol Med Microbiol 2000; 27: 163–169.
6. Brennan M, Thomas DY, Whiteway M, Kavanagh K. Correlation between virulence of *Candida albicans* mutants in mice and *Galleria mellonella* larvae. FEMS Immunol Med Microbiol 2002; 34: 153–157.
7. Reeves EP, Messina CGM, Doyle S, Kavanagh K. Correlation of Gliotoxin Production and Virulence of *Aspergillus fumigatus* in *Galleria mellonella*. Mycopathologia 2004; 158: 73–79.
8. Dunphy GB, Oberholzer U, Whiteway M, Zakarian RJ, Boomer I. Virulence of *Candida albicans* mutants towards larval *Galleria mellonella* (Insecta, Lepidoptera, Galleridae). Can J Microbiol 2003; 49: 514–524.
9. Maerker C, Rohde M, Brakhage AA, Brock M. Methylcitrate synthase from *Aspergillus fumigatus*: Propionyl-CoA affects polyketide synthesis, growth and morphology of conidia. FEBS J 2005; 272: 3615–3630.
10. Mylonakis E, Moreno R, El Khoury JB, Idnurm A, Heitman J, Calderwood SB, Ausbel FM, Diener A. *Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis. Infect Immun 2005; 73: 3842–3850.
11. Bhabhra R, Miley MD, Mylonakis E, Boettner D, Fortwendel J, Panepinto JC, Postow M, Rhodes JC, Askew DS. Disruption of the *Aspergillus fumigatus* gene encoding nucleolar protein Cgr A impairs thermotolerant

growth and reduces virulence. Infect Immun 2004; 72: 4731–4740.

12. Hamamoto H, Kurokawa K, Kaito C, Kamurw K et al. Quantitative evaluation of the therapeutic effects of antibiotics using silkworm infected with human pathogenic microorganisms. Antimicrob Agents Chemother 2004; 48: 774–779.
13. Dunphy G, Morton D, Kropinski Chadwick A J. Pathogenicity of lipopolysaccharide mutants of *Pseudomonas aeruginosa* for larvae of *Galleria mellonella*: Bacterial properties associated with virulence. J Invertebr Pathol 1986; 47: 48–55.
14. Walters JB, Ratcliffe NA. Studies on the *in vivo* cellular reactions of insects: Fate of pathogenic and non-pathogenic bacteria in *Galleria mellonella* nodules. J Insect Physiol 1983; 29: 417–424.
15. Arumugam M, Romestand B, Torreilles J, Roch P. *In vitro* production of superoxide and nitric oxide (as nitrite and nitrate) by *Mytilus galloprovincialis* haemocytes upon incubation with PMA or laminarin or during yeast phagocytosis. Eur J Cell Biol 2000; 79: 513–519.
16. Erickson DL, Lines JL, Pesci EC, Venturi V, Storey DG. *Pseudomonas aeruginosa rel A* contributes to virulence in *Drosophila melanogaster*. Infect Immun 2004; 72: 5638–5645.
17. Boman HG, Hultmark D. Cell-free immunity in insects. Annu Rev Microbiol 1987; 41: 103–126.
18. Price CD, Ratcliffe NA. A reappraisal of insect haemocyte classification by the examination of blood from fifteen insect orders. Z Zellforsch Mikrosk Anat 1974; 147: 537–549.
19. Tojo S, Naganuma F, Arakawa K, Yokoo S. Involvement of both granular cells and plasmatocytes in phagocytic reactions in the greater wax moth, *Galleria mellonella*. J Insect Physiol 2000; 46: 1129–1135.
20. Walters JB, Ratcliffe NA. Studies on the *in vivo* cellular reactions of insects: Fate of pathogenic and non-pathogenic bacteria in *Galleria mellonella* nodules. J Insect Physiol 1983; 29: 417–424.
21. Meister M, Georgel P, Lemaitre B, Kappler C, Lagueux M, Reichhart JM, Hoffmann JA. Immune gene expression in Drosophila. In: Hoffmann JA, Janeway CA Jr, Natori SRG, eds. Phylogenetic Perspectives in Immunity: The Insect Host Defence, Landes Company, Austin, 1994: 167–181.
22. Samakovlis C, Kimbrell DA, Kylsten P, Engstrom A, Hultmark D. The immune response in *Drosophila*: Pattern of cecropin expression and biological activity. EMBO J 1990; 9: 2969–2976.
23. Ratcliffe NA. Cellular defence responses in insects: Unresolved problems. In: Bechage NE, Thompson SN, Federici BA, eds. Parasites and Pathogens of Insects, 1, Academic Press, San Diego, 1993: 579–604.
24. Nappi AJ, Vass E. Hydrogen peroxide production in immune-reactive *Drosophila melanogaster*. J Parasitol 1998; 84: 1150–1157.
25. St. Leger RJ, Screen SE, Shams-Pirzadeh B. Lack of host specialization in *Aspergillus flavus*. Appl Environ Microbiol 2000; 66: 320–324.

Address for correspondence: Dr. Kevin Kavanagh, Department of Biology, NUI Maynooth, Medical Mycology Unit National Institute for Cellular Biotechnology, Co. Kildare, Ireland
Phone: +353-1-7083859; Fax: +353-1-7083845
E-mail: kevin.kavanagh@NUIM.ie.