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Technical Focus

Galleria mellonella larvae as models for studying fungal virulence

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ABSTRACT

As a result of the strong structural and functional similarities between the innate immune system of mammals and the insect immune response, insects have been exploited for evaluating the virulence of fungal pathogens of humans and for assessing the efficacy of anti-fungal agents. There is a strong correlation between the results obtained using insects and mammals and insects have the added advantage of being cheap to purchase, give results in 24–48 h and are without the ethical and legal restrictions associated with the use of mammals. Larvae of *Galleria mellonella* are excellent *in vivo* models and have been used with a variety of fungi and anti-fungal agents. Factors affecting the use of *G. mellonella* larvae are described and examples of where these larvae have been utilized are discussed.

1. Insect models for studying microbial pathogens of mammals

Conventional *in vivo* assays to assess the efficacy of anti-microbial agents or to determine the virulence of fungal pathogens or mutants have traditionally relied upon the use of a variety of vertebrate species. Mice, rats, guinea pigs and birds have been widely used with a range of fungal pathogens and although these animals give results that can be directly applied to humans their cost and the desire to reduce their use has led to the development of alternative models. Due to the strong structural and functional similarities between the immune response of insects and the innate immune responses of mammals insects are now being employed to study alterations in microbial virulence (Kavanagh and Reeves, 2004; Lionakis and Kontoyiannis, 2005; Fuchs and Mylonakis, 2006) and the efficacy of novel anti-microbial drugs (Hamamoto *et al.*, 2004, 2009). A variety of different insects species have been employed to study microbe–host interactions and include *Drosophila melanogaster*, *Galleria mellonella*, *Bombyx mori* and *Manduca sexta*. A wide range of microorganisms have been studied in insects including many bacterial

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(Dunphy *et al.*, 1986; Morton *et al.*, 1987; Bergin *et al.*, 2005) and fungal pathogens (Mylonakis *et al.*, 2005; Reeves *et al.*, 2004; Cotter *et al.*, 2000).

The insect immune response

The insect immune system demonstrates a number of structural and functional similarities to the innate immune response of mammals (Rock *et al.*, 1998; Wittwer *et al.*, 1999; Zhao and Kanost, 1996). The cuticle of the insect serves a function analogous to the skin of mammals and provides a barrier to infection. The haemolymph of insects is contained within the body cavity (haemocoel) and serves a function comparable to blood where it transports nutrients, waste products and signal molecules (Matha and Mracek, 1984). Haemolymph contains immune cells called haemocytes which are capable of immobilising and killing invading microbes (Lavine and Strand, 2002). There are at least six types of haemocytes in insects and these show similarities to mammalian neutrophils in terms of their ability to phagocytose and kill pathogens by the generation of superoxide (Bergin *et al.*, 2005; Renwick *et al.*, 2007). The humoral element of the insect immune response consists of the processes of melanisation, haemolymph clotting, anti-microbial peptide synthesis and wound healing (Kavanagh and Reeves, 2004). Anti-microbial peptides (AMPs) are released from haemocytes and internal organs into the haemolymph where they attack components of the bacterial and fungal cell wall (Ratcliffe, 1985). Since innate immune response of mammals is a vital component in the immune response to pathogenic infections (Levy, 2001; Romani, 1999), results obtained using insects show a strong correlation to those that can be obtained using mice (Brennan *et al.*, 2002).

Insect models of infection

Insect models of microbial infection have been employed to investigate the anti-microbial properties of drugs (Lionakis and Kontoyiannis, 2005; Johny *et al.*, 2007) and offer many advantages over the use of mammals (Kavanagh and Reeves, 2004). There have also been many reports of the efficacy of insects in studying the therapeutic effects of antibiotics (Hamamoto *et al.*, 2004; Alippi *et al.*, 2005) and the anti-microbial effects of various drugs (Lionakis *et al.*, 2005; Lionakis and Kontoyiannis, 2005; Tickoo and Russell, 2002). Lionakis and Kontoyiannis (2005) employed *D. melanogaster* as a fast, inexpensive high-throughput screening model for anti-*Aspergillus* compounds and in investigations of the role of *Aspergillus* virulence factors in pathogenesis. Although all *Aspergillus* virulence and therapeutic investigations cannot be performed in these models they do provide primary testing systems and may reduce the number of mammals being utilized.

2. The *G. mellonella* infection model

Recent studies have shown that larvae of the Greater Wax moth, *G. mellonella* (Lepidoptera: Pyralidae) may be used as effective models for studying microbial virulence *G. mellonella* larvae are easy to culture and are inexpensive to purchase (see Table 1). Results can be obtained within 48 h and a large amount of insects can be inoculated in a short period of time (Cotter *et al.*, 2000) (see Fig. 1). Larvae of *G. mellonella* have been employed to determine the relative virulence *Candida albicans* isolates and to differentiate between pathogenic and non-pathogenic yeast species (Cotter *et al.*, 2000). A positive correlation between the virulence of *C. albicans* mutants when tested in *G. mellonella* larvae and in BalbC mice has been established (Brennan *et al.*, 2002) and a number of factors affecting the virulence of *C. albicans* mutants in *Galleria* larvae have been identified (Dunphy *et al.*, 2003). *G. mellonella* larvae have been used to demonstrate a correlation between toxin production and virulence of the pulmonary pathogen *Aspergillus fumigatus* (Reeves *et al.*, 2004) and a correlation between the stage of *A. fumigatus* spore germination and virulence has also been established (Renwick *et al.*, 2006). Earlier work demonstrated the use of *Galleria* larvae for investigating the immunosuppressive activities of fungal secondary metabolites (Gotz *et al.*, 1997), by assessing anti-

Table 1 – Selected examples of utilization of *Galleria* larvae with fungi and anti-fungal agents

Yeasts	
Cotter *et al.*, 2000	Differentiation of virulence of range of yeast species
Brennan *et al.*, 2002	Comparison of virulence of *C. albicans* mutants with virulence in mice
Vegapuldi *et al.*, 2009	Sexually reproduced spores from *Crypotococcus* as effective infectious propagules
Mylonakis *et al.*, 2005	Development of model for studying virulence of *Cryptococcus*
Mylonakis *et al.*, 2005	Assessing the virulence of different *C. neoformans* strains in an invertebrate model
Fan *et al.*, 2007	Analysis of the virulence of Eca 1 mutants of *C. neoformans* in *G. mellonella*
Filamentous fungi	
Reeves *et al.*, 2006	Assessment of alteration in pes 1 mutant of *A. fumigatus*
Vickers *et al.*, 2007	Demonstration of efficacy of anti-Cts-D antibody *in vivo*
Wodja *et al.*, 2009	Heat shock and natural infection by *B. bassiana* induce an anti-fungal response
Jackson *et al.*, 2009	Assessment of virulence of melanin mutants of *A. fumigatus*
Scully and Bidochka, 2006	Host specialization of *A. flavus* in *G. mellonella*
Fuguet and Vey, 2004	Role of toxins from *Beauvaria* spp. in an *in vivo* model
Fuguet and Vey, 2004	Isolation and characterization of insecticidal chitinases
Reeves *et al.*, 2004	*In vivo* analysis of the effect of gliotoxin as a virulence factor of *A. fumigatus*
Assessment of anti-fungal activity	
Cowen *et al.*, 2009	Identification of Hsp 90 as a drug target in an *in vivo* model
Rowan *et al.*, 2009	Evaluation of anti-fungal efficacy of silver-based compounds

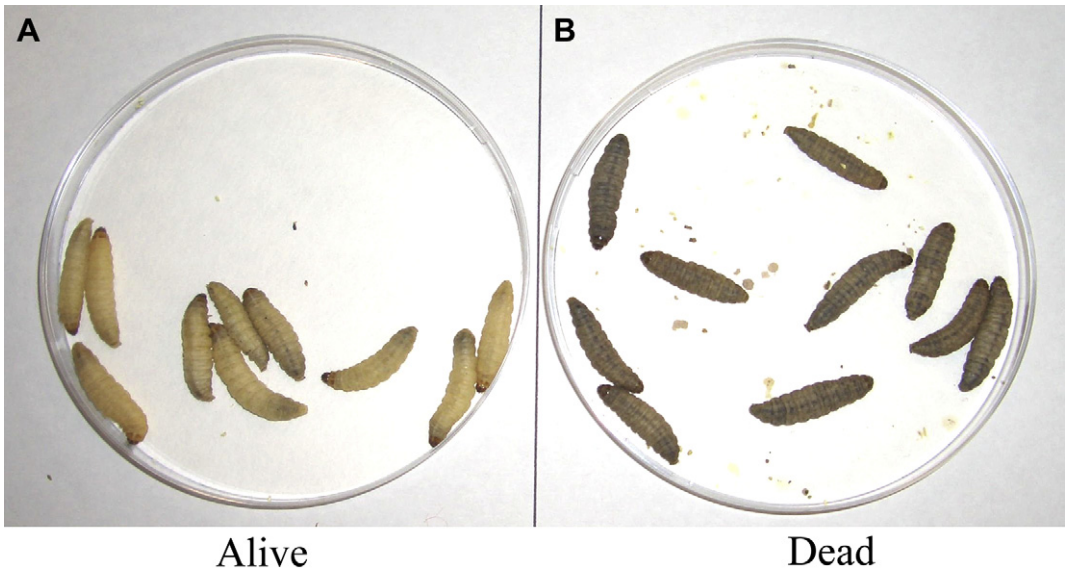

Fig. 1 – Living (A) and dead (B) *G. mellonella* larvae that had been infected with *C. albicans*.

phagocytic properties and changes in cytoskeletal microtubule formation.

G. mellonella larvae were also employed to establish appropriate infection models for *Cryptococcus neoformans* (Mylonakis *et al.*, 2005). Invertebrates such as *Acanthamoeba castellanii*, *Caenorhabditis elegans*, *Dictyostelium discoidium*, *D. melanogaster* and *G. mellonella* have been employed to study the molecular mechanisms by which *C. neoformans* interacts with the host (London *et al.*, 2006) and results revealed that several virulence-related genes previously known to be involved in *C. neoformans* mammalian infections also played a role in virulence in *G. mellonella*.

G. mellonella larvae have been employed to evaluate the anti-fungal activity of amphotericin B, flucytosine and fluconazole following infection with *C. neoformans* (Mylonakis *et al.*, 2005) and to evaluate the *in vivo* anti-fungal properties of novel silver-based compounds (Rowan *et al.*, 2009). When using *Galleria* larvae or other insects for assessing the anti-microbial properties of compounds it is important to note that the introduction of the agent into the haemocoel of the insect also provokes an anti-microbial immune response which operates in parallel with the anti-fungal activity of the introduced agent (Rowan *et al.*, 2009). Thus in cases where larvae are used to determine the anti-microbial properties of a novel compound it is important to be able to differentiate between the inherent anti-fungal properties of the agent and the anti-microbial responses induced by the agent.

3. Inoculation of *G. mellonella* larvae

While many insect models are now available for use the larvae of *G. mellonella* have many advantages. Due to their size (1.5–2.5 cm) it is possible to inoculate individual larvae with specific doses of the pathogen in question whereas it is difficult to quantify the dose per insect when using other models such as *Drosophila*. In addition *G. mellonella* larvae can be purchase commercially and yield results in 24–48 h. Larvae can be incubated at temperature up to 30–37 °C but appropriate controls should be employed as alterations in temperature can prime the immune response (Mowlds and Kavanagh, 2008).

Larvae of *G. mellonella* are easy to inoculate *via* injection into the haemocoel through the last left pro-leg (Cotter *et al.*, 2000) (see Fig. 2). The base of the pro-leg can be opened by applying gentle pressure to the sides of the leg and this aperture will re-seal after removal of the syringe needle without leaving a scar. Inoculation of larvae with test microorganisms must be accompanied by inoculation of larvae with the buffer used to re-suspend the test microorganisms (or to dissolve the test compound) to ensure that this has no impact on larval viability. A number of workers also suggest the 'mock-inoculation' of a number of larvae per experiment to ensure that the handling and inoculation procedures are not deleterious

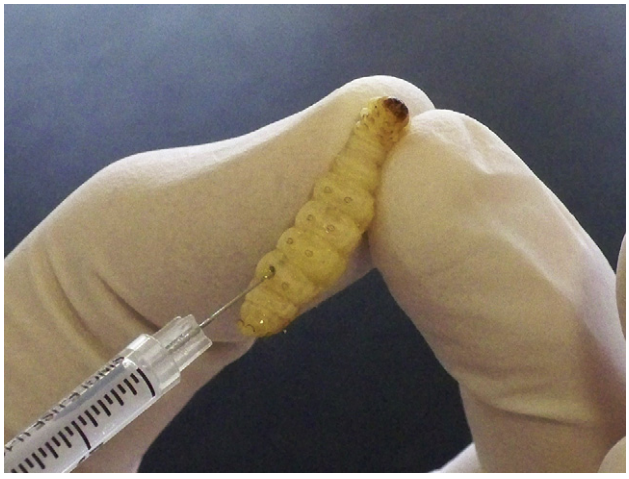

Fig. 2 – Procedure for inoculating *G. mellonella* larvae. The needle is inserted into the last left pro-leg in order to introduce the pathogen into the insect haemocoel.

to the health of the larvae (Dunphy and Webster, 1984; Cotter *et al*., 2000). Larvae can be stored at 15 °C prior to use and, once inoculated, may be maintained at temperatures up to 37 °C as long as appropriate controls are implemented to quantify the effect of temperature on survival. However the practice of 'chilling' larvae by incubation at 4 °C prior to inoculation in order to retard their movement should be avoided as it has been shown to alter their immune response and may compromise results (Mowlds and Kavanagh, 2008). Larvae should be handled with care as rough handling affects survival and also leads to the expression of stress proteins (Mowlds *et al*., 2008).

Inoculated larvae will begin to turn brown/black within a short period of time depending upon the virulence of the pathogen (Fig. 1). This is due to the onset of melanisation – an insect immune response resulting from the cleavage of prophenoloxidase to active phenoloxidase designed to limit the growth of the pathogen within the haemocoel. (Kavanagh and Reeves, 2004). Dead larvae will appear black in colour however it is worth noting that some pathogens (e.g. *Aspergillus flavus*) may lead to the appearance of ared/brown pigmentation in the insect cuticle. While larval death is often used as the endpoint in an experiment other parameters may also be employed particularly when dealing with a relatively 'weak' pathogen or mutant with attenuated virulence which may not actually kill the test larvae. Fluctuations in fungal load and haemocyte density have been used as accurate indicators of fungal virulence in larvae (Bergin *et al*., 2003) as have changes in the expression of anti-microbial peptides (Bergin *et al*., 2006). When analysing a variety of *C. albicans* strains in larvae of *G. mellonella* isolates of high virulence could be detected by a decrease in circulating haemocyte density and an increase in fungal load whereas isolates of low virulence were detectable by a high haemocyte density and a low fungal load (Bergin *et al*., 2003). Changes in the expression of selected anti-microbial peptides can be used as an indicator of the immune response to infection and assist in differentiating pathogenic from non-pathogenic infections. (Bergin *et al*., 2006). Irrespective of which endpoint is used (larval death, fungal load, haemocyte density, anti-microbial peptide expression, proteomic profile) results can be obtained within 48 h.

While *G. mellonella* larvae offer many advantages as an alternative model for studying fungal pathogens of humans a number of points should be borne in mind regarding the appropriateness of their use. The larvae are a good model for studying pathogens that infect systemically through the bloodstream (e.g. *C. albicans*, *C. neoformans*) and in our hands we have found the strongest correlation with the results obtained using BalbC mice (Brennan *et al*., 2002). Larvae do not possess comparable structures to many of the organs frequently infected by human fungal pathogens (e.g. lungs, spleen, brain) and as a consequence cannot be used as a model to study dissemination within these organs. However the use of *G. mellonella* larvae represents a significant reduction in time, cost and in the need to use mammals with the attendant ethical and legal concerns. Used correctly, and with the appropriate controls, insects (Kavanagh and Reeves, 2004) and *G. mellonella* larvae in particular (Mylonakis, 2008), provide a rapid and effective means of studying fungal virulence and for assessing the efficacy of anti-microbial drugs.

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