Mycopathologia (2008) 165:5–12
DOI 10.1007/s11046-007-9069-9

Effect of pre-incubation temperature on susceptibility of *Galleria mellonella* larvae to infection by *Candida albicans*

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Received: 15 August 2007 / Accepted: 19 September 2007 / Published online: 6 October 2007

Abstract The use of insects for evaluating the virulence of microbial pathogens and for determining the efficacy of antimicrobial drugs is increasing. When larvae of the greater wax moth *Galleria mellonella* were incubated at 4 or 37°C for 24 h. prior to infection, they manifested increased resistance to infection by the yeast *Candida albicans* compared to larvae that had been pre-incubated for 24 h at 30°C. Incubation at 4 or 37°C led to an increase in haemocyte density and the expression of genes coding for gallerimycin, transferrin, an inducible metalloproteinase inhibitor (IMPI) and galiomicin. Peak expression of these genes was recorded at approximately 24 h after the commencement of the 4 or 37°C incubation. These results indicate that exposure of larvae to mild thermal shock conditions induces a protective cellular and humoral immune response mediated by increased numbers of haemocytes and elevated expression of antimicrobial peptides.

Keywords Anti microbial peptides · Cellular response · *Galleria mellonella* · Gene expression · Haemocytes · Host defence · Humoral response

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Abbreviations
AMP Anti-microbial peptide(s)
IMPI Inducible metalloproteinase inhibitor

Introduction

The insect immune system demonstrates a number of structural and functional similarities to the innate immune system of mammals [1] and, as a consequence, insects can be utilised as models for assessing the virulence of a variety of microbial pathogens [2, 3]. A wide range of insects are now employed to assess microbial virulence or to determine the antimicrobial activity of drugs. Larvae of the greater wax moth *Galleria mellonella* have been shown to be an efficient model for studying the in vivo pathogenicity of yeasts [4], mutants of *Candida albicans* [5] and the virulence of *Aspergillus fumigatus* [6].

The immune response of insects consist of a cooperative functioning system with both humoral and cellular components. The cellular immune response consists of the rapid synthesis and mobliisation of immune cells called haemocytes, which engulf or surround invading pathogens. Six types of haemocytes have been classified [7] and include plasmatocytes, granulocytes, prohaemocytes, coagulocytes, spheruloctes and oenocytoids [8]. The humoral element of the insect immune response consists of the production of antimicrobial peptides

(AMP) which are a primitive yet competent mechanism of defence found in vertebrates, insect and plants [9, 10]. AMPs are mostly small, less than 10 kDa in size, and hydrophobic membrane active peptides. These peptides alter the cell membrane of pathogenic microorganisms and recognise the acidic phospholipids exposed on the surface of the bacterial membrane [11]. AMPs found in *Galleria mellonella* include inducible metalloproteinase inhibitor (IPMI), transferrin, galiomicin and gallerimycin and these play an important role in the insect humoral response [12]. Without the signal dependent synthesis of AMPs insects would succumb quickly to microbial infection [13]. It has been previously demonstrated that *G. mellonella* larvae pre-inoculated with a sub-lethal dose of the yeast *Candida albicans* or fungal cell wall components demonstrate an increased expression of the antimicrobial peptides and are capable of dealing with a potentially lethal doses of *C. albicans* [12].

The aim of the work presented here was to establish the effect of incubation temperature on the immune response of *G. mellonella* to infection by the yeast *C. albicans*. Incubation temperature can significantly affect insect physiology and behaviour [14] and it was hoped to establish how temperature altered the cellular and immune responses of *G. mellonella* larvae, so that optimal condition for the use of this insect in in vivo assays could be determined.

Material and methods

Chemicals

All Chemicals and reagents were of the highest quality and purity and unless otherwise stated were purchased from Sigma Aldrich Chemical Co. Ltd, Dorset, UK.

Yeast strains and culture conditions

The yeast strain used in this study was *Candida albicans* MEN (a generous gift from Dr. D. Kerridge, Cambridge, UK). Yeast cultures were grown to the stationary phase (approximately 1×10^8/ml) in 50 ml of YEPD broth (2% (w/v) glucose, 2% (w/v) bactopeptone (Oxoid Ltd., Basingstoke, England) and 1% (w/v) yeast extract (Oxoid)) in 100 ml conical flasks at 30°C and 200 rpm in an orbital incubator.

Insect larvae

Sixth instar larvae of *G. mellonella* (Lepidoptera: Pyralidae, the Greater Wax Moth)(Mealworm Company, Sheffield, England) were stored in wood shavings in the dark at 15°C and larvae were inoculated as described previously [4]. Only larvae weighing between 0.2 and 0.4 g were used during this study.

Pre-incubation of insect larvae

Three thermal treatments were utilised in this work. All larvae were stored at 15°C initially but in the 24 h immediately prior to infection with *C. albicans* larvae were maintained at 4, 37 or 30°C in the dark and then incubated, following inoculation, at 30°C. Ten larvae were used per treatment and all experiments were performed on three independent occasions.

Determination of haemocyte density

Haemocyte density was assessed by bleeding three larvae into a pre-chilled microcentrifuge tube containing a few granules of phenylthiourea to prevent melanisation, as described [15]. Dilutions were made by mixing the haemolymph with 0.37% (v/v) mercaptoethanol in sterile PBS. Haemocyte density was assessed with the aid of a Neubauer haemocytometer.

Analysis of expression of immune relevant proteins of *G. mellonella* by RT-PCR

A TRI-reagent method was used for the extraction of RNA from larvae incubated at 4, 30 or 37°C for various periods of time. About 2 μg of RNA was used for the synthesis of cDNA using a SuperScript Kit (Introvogen) with oligo (dT) primers. RNA was treated with DNase I prior to cDNA synthesis. Amplification of target genes was performed using

primers that are previously described [12] and using the following cycle conditions: 98°C denaturation for 2 min (94°C denaturation for 60 s, 55°C for 60 s, 72°C extension for 90 s) × 30 cycles; 72°C extension for 10 min. Amplified products were visualised on a 1% Agarose gel containing ethidium bromide after which a Sygene Geneflash and densitometric analysis of PCR products was carried out using Imagequant software. All samples were normalised against β actin values. The highest level of expression was set to 100% and other values are given as a percentage of relative activity [16].

Statistical analysis

All assays were preformed on three independent occasions. Results are expressed as the mean ± SE and were compared by *t*-test using Sigma Stat Statistical analysis Package Version 1.00 (SPSS Inc, Chicago, IL, USA). Differences were considered significant at $P \leq 0.05$.

Results

Effect of pre-incubation temperature on larval survival following microbial challenge

It has been established, previously, that exposure of *Galleria* larvae to non-lethal doses of yeast or fungal cell wall constituents protects against lethal doses of *C. albicans* [12]. In the work presented here, we sought to establish, whether temperature influenced larval survival. Larvae of *G. mellonella* were incubated at 4, 37 or 30°C for 24 h, as described, and inoculated with 5×10^5 *C. albicans* cells per larva. All larvae were subsequently incubated at 30°C and viability was monitored over a 72 h period. Incubation at the different temperature for 24 h prior to infection did not adversely affect larval survival. The results (Fig. 1) indicate that in larvae pre-incubated at 30°C viability decreased to 23 ± 3.3% after 24 h after infection and to 10 ± 0% after 72 h. In contrast, prior exposure of larvae to a temperature of 37°C or 4°C ensured that 73 ± 5.7% and 83 ± 12% of larvae, respectively, were still alive after 24 h and that larval survival following these treatments is approximately 50% at 72 h.

Effect of incubation temperature on larval haemocyte density

Haemocytes are immune cells that constitute the cellular arm of the insect immune response to pathogens and elevated levels of haemocytes have been previously associated with a protective response against microbial infections [15, 17, 18]. In order to establish whether a change in circulating haemocyte density was responsible for the protective effect observed in larvae pre-incubated at different temperatures haemolymph samples were isolated the number of haemocytes enumerated. Larvae that had been pre-incubated at 30°C prior to infection (Fig. 2) showed a haemocyte density of 3.2×10^5/larva at 24 h. Exposure of larvae to a temperature of 4°C increased haemocyte density to approximately 5.4×10^5 haemocytes/larva at 24 and 48 h. Exposure of larvae to 37°C resulted in a dramatic increase in haemocyte density which reached 9.5×10^5/larva after 24 h and 8.7×10^5/larva after 48 h. These results indicate that incubation temperature can induce an increase in haemocyte density that could contribute to the protective effective evident following *C. albicans* challenge.

Gene expression of proteins involved in an immune response

It has been previously established that exposure of *Galleria* larvae to a non-lethal dose of *C. albicans, Saccharomyces cerevisiae* or fungal cell wall constituents protects larvae against a subsequent lethal inoculation of *C. albicans* cells and that this protection is mediated by the increased expression of selected antimicrobial peptides [12]. The expression of selected immune proteins has also been shown to be increased in *Drosophila* during an immune response [16].

The expression of genes coding for selected antimicrobial peptides was examined in order to establish whether incubation temperature affected their expression in larvae. Larvae were exposed to the different temperature regimes, as described, and the gene expression of four AMPs was monitored at 1, 4, 18, 24 and 48 h (Fig. 3). It should be noted that inoculation normally occurred after the 24 h of incubation at the three test temperature. The genes

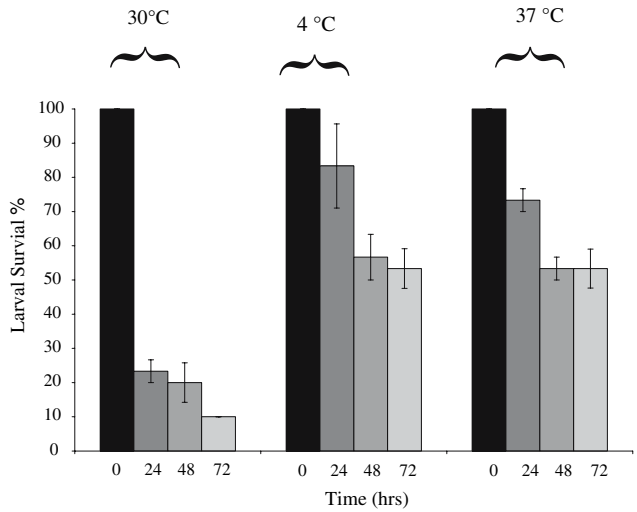

Fig. 1 Viability of *Galleria* larvae inoculated with *C. albicans* following different thermal treatments. Larvae were pre-incubated 4, 30 or 37°C for 24 h prior to infection with *C. albicans*. Infected larvae were subsequently incubated at 30°C. The percentage survival at 0, 24, 48 and 72 h is recorded and all values represent the mean ± standard error from three independent experiments

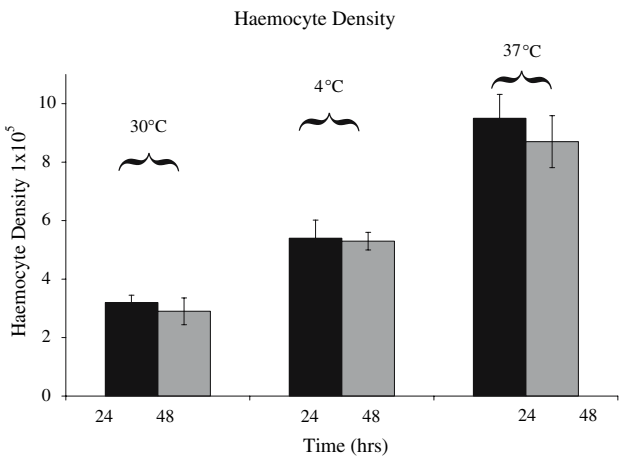

Fig. 2 The effect of incubation temperature on the haemocyte density of *Galleria* larvae. Haemolymph was bled from insects that were incubated at 4, 30 or 37°C and the density of haemocytes per larva was calculated. All values represent the mean ± standard error from three independent experiments

that were examined coded for galiomicin, a defensin identified in *G. mellonella* [19], a cysteine rich antifungal peptide gallerimycin [20], transferrin, an iron binding protein [21] and an inducible metallo-proteinase inhibitor (IMPI) from *G. mellonella* that acts by inhibiting enzymes secreted by pathogenic microbes [22, 23].

Larvae demonstrated increased expression of galiomicin, which peaked at 24–48 h following each temperature treatment (Fig. 3 and 4). In larvae pre-incubated at 4°C peak expression was recorded at 48 h while in those larvae pre-incubated at 37°C peak expression was evident at 24 h. In general the highest levels of expression were recorded in those larvae that had been incubated at 4 or 37°C. Gallerimycin gene expression levels rose dramatically in the first 4 h of the analysis in all the larvae and reached levels approximately twice those recorded at 0 h for larvae pre-exposed to 4°C. The highest expression levels of gallerimycin for the different treatments were recorded at 24 h. (Fig. 4).

There was a small increase in expression of IMPI gene following incubation of larvae at 37°C and a dramatic drop at 48 h. In the case of larvae pre-exposed to 4°C expression increases until 24 h and then declines. Larvae pre-exposed to 30°C increased expression but this increase is not as dramatic as following the other treatments (Fig. 4). Transferrin expression in all larvae is greatest at 24 h (Fig. 4) and initial levels in the three treatment regimes are similar but the elevation is greatest in those larvae pre-exposed to 4 or 37°C.

The results presented here indicate that exposure of larvae to thermal stress, by incubation at 4 or 37°C,

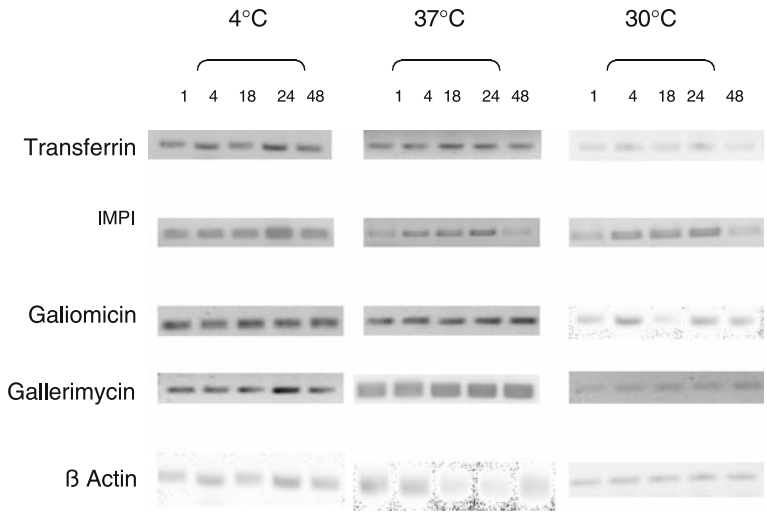

Fig. 3 RT-PCR analysis of *G. mellonella* cDNA from whole larvae on a 1% agarose gel. Larvae were incubated at 4, 30 or 37°C for various periods of time. PCR was performed using primers for galiomicin, gallerimycin, transferrin, IMPI and β actin (housekeeping gene) on cDNA

induces increased expression of genes coding for key anti-microbial peptides and that in many cases peak expression is at 24 h which coincides with the administration of the potentially lethal *C. albicans* inoculation.

Discussion

There is an increasing awareness of the structural and functional similarities of the insect immune system and the innate immune system of mammals. Since the innate immune system of mammals represents the key response to microbial pathogens results obtained using insects can correlate with equivalent mammalian responses [5, 24]. This fact has been exploited through the use of insects for evaluating the virulence of microbial pathogens and for determining the efficacy of antimicrobial drugs prior to testing in mammals [2, 3]. A wide variety of insects are now employed as models for in vivo pathogenicity testing and have been used for assessing the virulence of yeast [4], mutants of *C. albicans* [5], *Cryptococcus neoformans* [25], *A. fumigatus* [6] and in testing antimicrobial drugs [26]. We have previously demonstrated the increased survival of larvae of *G. mellonella* to infection by *C. albicans* by first exposing larvae to non-lethal levels of yeast or fungal cell wall constituents [12].

The work presented here indicates that exposure of larvae to different incubation temperatures prior to inoculation with *C. albicans* can significantly affect the insect immune response. Prior exposure of larvae to temperatures of 4 or 37°C increased survival following infection with *C. albicans* and this appears to be mediated by an elevation in the haemocyte density and in the expression of a number of antimicrobial peptides. Increased haemocytes density has been previously associated with protection of larvae from microbial pathogens [15, 17, 18]. In general, low haemocyte numbers equate with low survival rates (larvae that had been pre-incubated at 30°C for 24 h showed 3.2×10^5 haemocytes/larva and a survival rate of 23.3 ± 3.3%), while high haemocyte numbers occurred with high survival rates (larvae that had been pre-incubated at 4°C had a haemocyte density of 5.4×10^5/larva of and a survival rate of 83 ± 12%) although this effect should not be seen in isolation as higher haemocyte numbers were observed in the larvae pre-incubated at 37°C but gave a lower survival rate (73.5 ± 5.7) to that seen in those larvae incubated at 4°C. It should be noted (Fig. 4) that the greatest expression of antimicrobial peptides was recorded in larvae pre-incubate at 4°C and this together with the elevated haemocyte density is probably affecting larval survival.

Anti-microbial peptide production is part of the humoral component of the insect immune response and the main sites of synthesis of anti-microbial peptides in the insect are the fat body, haemocytes, the digestive tract, salivary glands and the reproductive tract. Anti-microbial peptides represent the last line of defence and are released into the haemolymph where they attack elements of the bacterial or fungal

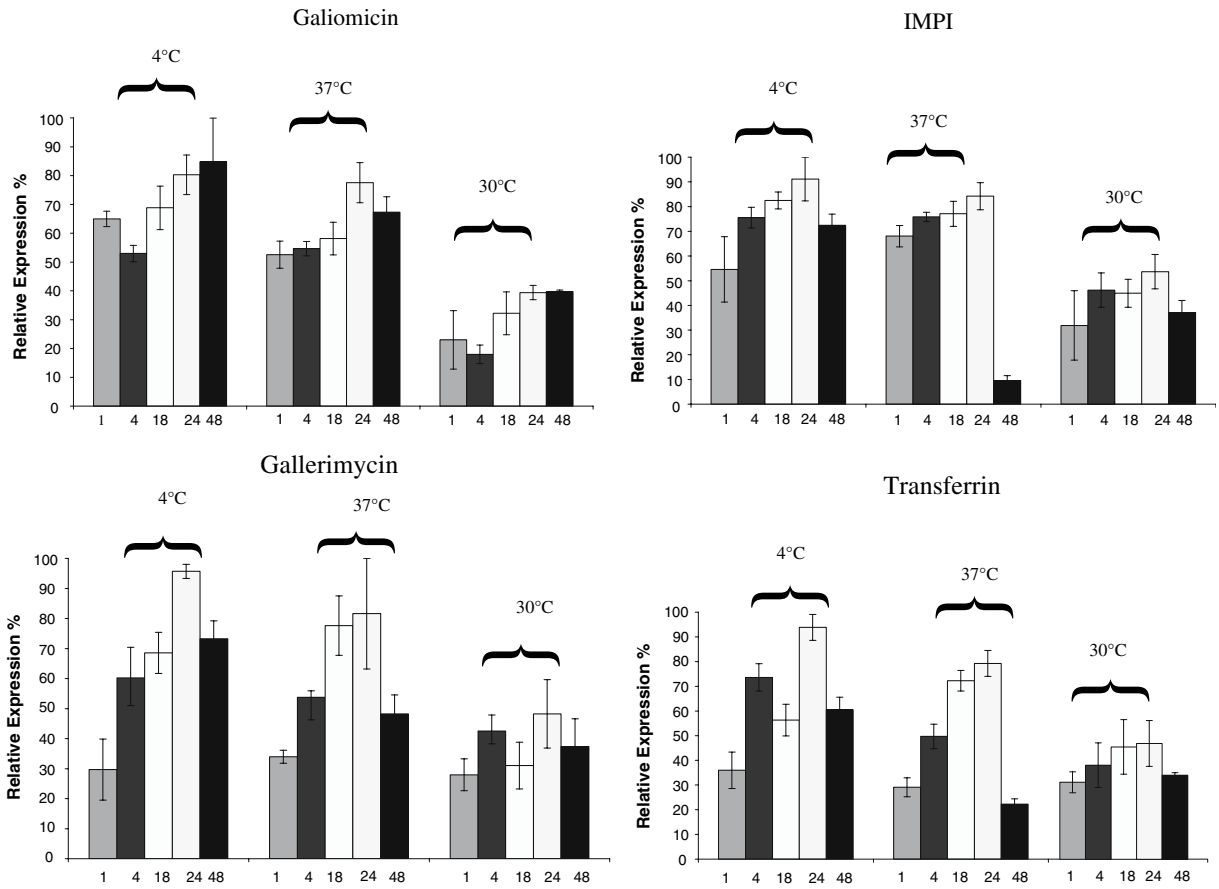

Fig. 4 Quantification of RT-PCR from larvae incubated at 4, 30 or 37°C. Densitometric quantification of PCR products from unsaturated images of RT-PCR was performed using Genetools software (Syngene). Values were then normalised with the corresponding value of β actin. The treatment that gave the highest level of expression was normalised to 100 and the remaining results are expressed as relative expression (%)

cell wall [27]. Anti-microbial peptides are synthesised as pre-proproteins at a rate up to 100 times faster than IgM in mammals [28] and their small size allows diffusion through the haemolymph to counteract invading pathogens. Such peptides play a crucial role in combating infection and similar classes of peptides are found in vertebrates, invertebrates, and plants [29].

Analysis of the expression of genes coding for selected antimicrobial peptides revealed that incubation temperature significantly affects expression and that in many cases peak expression was evident at 24 h, which coincided with the time larvae, were challenged with *C. albicans*. Galiomicin demonstrates antibacterial and antifungal effects [19] and increased expression has been recorded in *G. mellonella* larvae inoculated with *E. coli*. In the work presented here galiomicin expression peaks at 24–48 h for those larvae pre-exposed to 4°C and at 24 h for those pre-incubated at 37°C. Gallerimycin is a cysteine rich antimicrobial peptide and expression increases when larvae are inoculated with LPS [20]. Elevated expression levels of gallerimycin were recorded at all time points and peak expression was evident at 24 h in the three treatment groups. In insects transferrin has an iron-binding domain in the N-terminal region and may function by sequestering iron from pathogens thus inhibiting their growth [21]. Transferrin expression in *D. melanogaster* is up regulated upon infection with bacteria [21]. In the work presented here transferrin expression levels show a dramatic increase in those larvae pre-incubated at 4 and 37°C while levels show a smaller increase in those larvae pre-incubated at 30°C. Inducible metalloproteinase inhibitor (IMPI) was the

first metalloproteinase inhibitor characterised in invertebrates and was purified from *G. mellonella*. IMPI has well-established antifungal properties [22] and acts by inhibiting the action of enzymes secreted by pathogenic microbes [23]. Peak expression levels of IMPI are achieved at 24 h in larvae subjected to the three temperature treatments.

The data presented here indicate that incubation temperature has a very strong effect on the immune response of *G. mellonella* larvae. Exposure of larvae to 4 or 37°C leads to increased survival following challenge with *C. albicans* and this protective response appears to be mediated by increased numbers of circulating haemocytes and by the elevated expression of genes coding for a range of antimicrobial peptides. Overall, pre-incubation of larvae at 4 or 37°C leads to greater levels of expression of antimicrobial peptides than pre-incubation at 30°C possibly indicating that the insect finds the higher (37°C) and lower (4°C) temperatures stressful [30]. Given the increased use of insects as models for in vivo testing of microbial pathogens and evaluation of antimicrobial drugs [2, 3] these findings indicate that the incubation temperature can significantly affect the larval immune response and thus fluctuations should be minimised to ensure consistency of results.

Acknowledgements This work was supported by funding from the Higher Education Authority of Ireland through the Programme for Research in Third Level Institutes III (2002–2007).

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