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Original article

Pre-exposure to yeast protects larvae of *Galleria mellonella* from a subsequent lethal infection by *Candida albicans* and is mediated by the increased expression of antimicrobial peptides

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Abstract

Pre-exposure of the larvae of *Galleria mellonella* to *Candida albicans* or *Saccharomyces cerevisiae* protects against a subsequent infection with 10^6 *C. albicans* cells. This protection can also be induced by exposing larvae to glucan or laminarin prior to the administration of the potentially lethal inoculum. Analysis of the genes coding for *galiomicin*, a defensin in *G. mellonella*, a cysteine-rich antifungal peptide *gallerimycin*, an iron-binding protein *transferrin* and an inducible metalloproteinase inhibitor (IMPI) from *G. mellonella* demonstrated increased expression, which is at its highest after 24 h of the initial inoculum. Examination of the expression of proteins in the insect haemolymph using 2D electrophoresis and MALDI TOF analysis revealed an increased expression of a number of proteins associated with the insect immune response to infection 24 h after the initial exposure. This study demonstrates that the larvae of *G. mellonella* can withstand a lethal inoculum of *C. albicans* if pre-exposed to a non-lethal dose of yeast or polysaccharide 24 h previously which is mediated by increased expression of a number of antimicrobial peptides and the appearance of a number of peptides in the challenged larvae.

Keywords: *Galleria mellonella*; Antimicrobial proteins; Host defence; 2D gel electrophoresis; MALDI TOF analysis; Gene expression

1. Introduction

Insects are one of the most successful and diverse forms of animal life on Earth [1] and possess an immune system that shows strong structural and functional similarities with the innate immune system of mammals [2]. During an infection, the insect's immune response involves a cellular component where haemocytes recognise and phagocytose microbes, form nodules or encapsulate foreign particles [3]. The humoral element of the immune response consists of proteins involved in clotting such as vitellogenin-like proteins that contain a cysteine-rich region which is homologous to the mammalian clottable proteins of the Von Willebrand factor involved in blood clotting [2], and antimicrobial peptides (AMPs) such as defensins, which have been highly conserved through evolution [4]. AMPs are released from a range of organs and cells [5,6] into the haemolymph of the insect where they diffuse to the site of infection and attack components of the bacterial or fungal cell wall [7]. Haemocytes, the fat body and the digestive tract secrete antimicrobial proteins and peptides into the insect haemolymph, which performs many functions analogous to mammalian serum [5,6,8]. The similarity of a range of insect immune responses with vertebrate innate immune responses to infection has been highlighted by the discovery of the Toll receptors in insects and their similarity with the toll like receptors (TLR) in mammals and 11 members of this family have been identified in humans [9].

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RNA analysis or Reverse Transcriptase Polymerase Chain Reaction (RT PCR) has been employed to quantify transcript levels of specific genes. Whole RNA from adult *Drosophila* infected with a range of microorganisms was examined using Northern blotting and established that several antimicrobial peptide transcripts were differentially expressed over 72 h depending upon the microbe used in the infection study [10]. Differential expression of transferrin [11] and a metalloproteinase inhibitor in *Galleria mellonella* following exposure to LPS has also been observed [12].

Mass spectrometry analysis of tryptic-digested proteins or naturally occurring peptides (peptidomics) has also been used to quantify the changes in protein expression and the induction of novel proteins following infection [13]. Recent studies have utilised two-dimensional (2D) analysis of *Drosophila* haemolymph and *Anopheles gambiae* [14] and many groups have reported data concerning protein expression and induction in *Drosophila* utilising a proteomic approach [15–17].

Given the similarities between the insect immune response and the innate immune response of mammals, insects have been utilised to quantify the pathogenicity of microbes and to model the innate immune response without the requirement of mammals [18]. Insects have been employed to assess the relative pathogenicity of bacteria [19], fungi [20] and parasites [21] and positive correlations with results from murine studies have been demonstrated in *C. albicans* [22] and *Pseudomonas aeruginosa* [23].

The aim of the work presented here was to establish whether it was possible to induce a protective immune response in the larvae of *G. mellonella* following infection with a sub-lethal dose of yeast or fungal cell wall components. It was our intention to establish how this protection was induced in the insects and to ascertain the nature of the peptides mediating the effect.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were of the highest purity and were purchased from Sigma Aldrich Chemical Company Ltd. (Dorset, UK) unless stated otherwise.

2.2. Yeast strains and culture conditions

C. albicans MEN (Dr. D. Kerridge, Cambridge, UK) and *Saccharomyces cerevisiae* YJM128 (Dr. K. Clemons, Santa Clara Valley Medical Centre, CA, USA) were used in this study. Yeast cultures were grown to the stationary phase (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.

2.3. 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 [20]. Larvae were inoculated as described previously [20].

2.4. Induction of expression of immune relevant proteins of G. mellonella by RT PCR

Larval RNA was extracted using TRI-reagent at 1, 4, 8, 24 and 48 h post-infection. RNA (2 μg) was treated with DNase I prior to cDNA synthesis using the SuperScript Kit (Invitrogen) with oligo(dT) primers.

PCR amplification of target genes was performed with primers listed in Table 1 and using the following conditions: 94 °C denaturation for 5 min (94 °C denaturation for 60 s, 55 °C for 90 s, 68 °C extension for 90 s) × 26 cycles; 68 °C extension for 10 min. Visualisation of amplified products was performed using a Syngene Geneflash and densitometric analysis of PCR products was carried out using Genetools software. All samples were normalised to the corresponding β actin value. The highest level of expression in a series was set to 100% and other values of that series are given as percentage relative activity [10].

2.5. 2D gel electrophoretic separation of haemolymph proteins

Iso-electric focussing (IEF) was performed with 0.3 mg of haemolymph protein loaded on 13 cm IPG strips (Amersham Biosciences UK Ltd.) with 50 μA per strip and using the IPGphor focusing system (Amersham Biosciences) with the following running conditions: 10 h at 50 V, 15 min at 250 V, 5 h gradient at 8000 V and the final step was 8 h step and hold at 8000 V.

After separation of proteins in the first dimension, strips were equilibrated twice for 15 min in equilibration buffer

Table 1
PCR primer pairs used to amplify regions of four genes involved in the immune system and a housekeeping gene

Primer name	Oligonucleotides	Fragment size (base pair (bp))
β actin F[a]	GGGACGATATGGAGAAGATCTG	400
β actin R[b]	CACGCTCTGTGAGGATCTTC	
Transferrin F	CCCGAAGATGAACGATCAC	535
Transferrin R	CGAAAGGCCTAGAACGTTTG	
IMPI F	ATTTGTAACGGTGGACACGA	409
IMPI R	CGCAAATTGGTATGCATGG	
Galiomicin F	CCTCTGATTGCAATGCTGAGTG	359
Galiomicin R	GCTGCCAAGTTAGTCAACAGG	
Gallerimycin F	GAAGATCGCTTTCATAGTCGC	175
Gallerimycin R	TACTCCTGCAGTTAGCAATGC	

[a] F indicates a forward primer.
[b] R indicates a reverse primer.