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Original article

Dose-dependent cellular and humoral responses in *Galleria mellonella* larvae following β-glucan inoculation

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Abstract

Galleria mellonella larvae were inoculated with different doses of β-glucan by injection into the haemocoel. Those larvae that had received high doses of β-glucan (15, 30 or 60 μg/larva) demonstrated increased survival following infection with the yeast *Candida albicans*. High concentrations of glucan induced an increase in haemocyte density and a reduction in yeast proliferation within the haemocoel. Proteomic analysis of glucan-treated larvae revealed increased expression of a variety of peptides some of which may possess antimicrobial properties. Analysis of expression profiles revealed that low doses of β-glucan (3.75 μg/larva) triggered the increased expression of certain peptides (e.g. hemolin) while high dose inoculation was required before the increased expression of others (e.g. archaemetzincin) was evident. These results indicate that low doses of β-glucan induce a limited immune response while high doses induce an immune response that has the potential to curtail the threat within the haemocoel but also withstand a subsequent infection. Immune priming gives insects the ability to withstand a potentially lethal infection if exposed to a low level of the pathogen 24–48 h previously. Immune priming has resource implications and this work indicates that a graded immune response is initiated depending upon the amount of the immune priming agent encountered.

Keywords: Antimicrobial peptides; *Galleria* larvae; Haemocytes; Insect; Immune response; Proteomics

1. Introduction

Insects are an extremely successful group of organisms and have exploited almost all habitats on earth with the exception of the polar regions and the oceans. Their success is partly due to their high rates of reproduction but also to the possession of a rapidly mobilized and effective immune response that protects them from a variety of microbial pathogens and parasites. The insect immune system consists of interconnecting cellular [1] and humoral responses [2] which can deal quickly and successfully with agents that breach the cuticle. The cellular responses are mediated by haemocytes which are found in the insect haemolymph and operate by engulfing or binding to foreign cells that breach the cuticle [3]. The density of haemocytes circulating in the haemolymph may be indicative of infection, with low haemocyte densities being associated with infection and high haemocyte densities associated with healthy insects [4]. 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 [1], and antimicrobial peptides (AMPs) such as defensins, which have been highly conserved through evolution [5]. AMPs are released by a number of cells and organs [6,7] and attack elements of the bacterial or fungal cell that have entered the haemolymph [8].

The task of recognition of micro-organisms or non-self material that breach the cuticle and the activation of the appropriate pathways is achieved by pattern recognition receptors (PRRs)[2]. Many PRRs exist in insects such as LPS binding proteins [9], β-1,3 glucan binding protein [10], Gram-negative bacterial recognition protein [9] and peptidoglycan

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recognition protein [11]. Two distinct members of the NF-κβ family of inducible transactivators, DIF (dorsal-related immunity factor) and Relish are responsible for the regulation of antimicrobial peptide production [12–14].

Studies in *Drosophila* that examined the response to fungal or bacterial pathogens have revealed that insects can modulate their immune response to various classes of micro-organisms with the induction of selected antimicrobial peptide genes [13]. Previous work has indicated that administration of fungal cell wall components to *Galleria mellonella* larvae leads to an enhanced immune response and elevated resistance to infection with the yeast *Candida albicans* [15]. Such immune priming in insects is characterized by the ability of the insect to withstand a potentially lethal infection if a small sub-lethal inoculum is encountered a short time previously. This has obvious survival benefits but does impose a cost on the insect. The aim of the work presented here was to characterize the immune response of *G. mellonella* (Lepidoptera: Pyralidae, the Greater Wax Moth) larvae to different doses of glucan and to establish whether a differential response was evident in terms of changes in the density of circulating haemocytes, alterations in yeast replication within larvae and in the expression of selected antimicrobial peptides. Larvae of *G. mellonella* are useful models for assessing the virulence of human fungal pathogens [4] since the insect immune response manifests a number of similarities with the innate immune response of mammals. It was hoped to establish whether there exists a critical level of glucan above which a comprehensive immune response, in terms of elevation in haemocyte density and increased expression of antimicrobial peptides, is initiated.

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

Candida albicans MEN (kindly donated by Dr. D. Kerridge, Cambridge, UK) was 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.

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. Larvae that weighed between 0.2 and 0.4 g were used in all experiments. Larvae were inoculated with different concentrations of β-glucan, (0.93, 1.87, 3.75, 7.5, 15, 30 or 60 μg/larva) dissolved in PBS 24 h prior to infection with *C. albicans* (1×10^6/larva) as described previously [16]. Controls consisted of untouched larvae or those that received a (20 μl) PBS inoculation. Ten larvae were used per treatment, with all treatments being performed on three independent occasions.

2.4. Determination of haemocyte density

Larvae were inoculated with different concentrations of β-glucan, (0.93, 1.87, 3.75, 7.5, 15, 30 or 60 μg/larva) and the haemocyte density was assessed by bleeding three larvae 24 or 48 h after administration of glucan into a pre-chilled microcentrifuge tube containing a few granules of phenylthiourea to prevent melanisation. Dilutions were made by mixing the haemolymph with 0.37% (v/v) mercaptoethanol in sterile PBS and haemocyte density was assessed with the aid of a Neubauer haemocytometer [4]. All determinations were performed on three independent occasions.

2.5. Determination of fungal load in G. mellonella larvae

Three larvae that had been inoculated with cells of *C. albicans,* with or without prior β-glucan inoculation, were homogenized in 3 ml of sterile PBS. This was serially diluted with PBS and 100 μl aliquots of the resulting dilutions were plated on YEPD plates containing erythromycin (1 mg/ml) to prevent bacterial growth. Plates were incubated at 30 °C for 48 h. The fungal load was calculated as the yeast cell density per larva and was based on the number of colonies that grew at specific dilutions [4].

2.6. 2D gel electrophoretic separation of haemolymph proteins and image analysis

Haemolymph (100 μl) was collected from larvae that had been inoculated with glucan 24 h previously by piercing the head of the insect and bleeding the haemolymph into a pre-chilled microcentrifuge tube. Iso-electric focusing of protein samples and 2D electrophoresis was performed as described previously [17]. Each 2D gel was scanned on a Hewlett Packard scanjet 5100c scanner and the images analysed with ImageMaster 2D Platinum v7 software (Amersham Biosciences UK Ltd.). The protein spots of interest on each gel were detected, normalised, edited and manually matched to a reference gel. Proteins separated by 2D electrophoresis were quantified in terms of their relative volume (% Vol). The intensity volume of each spot was processed by background subtraction and total spot volume normalization, and the resulting spot volume percentage was used for comparison.

2.7. Protein identification by MALDI-TOF analysis

Mass spectrometry of trypsin digested proteins was performed using an Ettan™ MALDI-TOF spectrometer (Amersham Biosciences, Gmbh, Freiburg, Germany) as described previously [17]. Resulting mass lists from tryptic-digested protein were analysed using ProFound peptide mapping version 4.10.5 developed by Rockefeller University (http://

www.unb.br/cbsp/paginiciais/profound.htm). The taxonomy used to identify tryptic fingerprint was *Drosophila* and other metazoa with a tolerance mass error of 1.0 Da. Verification of sequences performed using a BLAST search on the NCBI website (http://www.ncbi.nlm.nih.gov) to identify conserved domains of protein families. A z score of 1.65 is considered to be 95% confident.

2.8. Statistical analysis

All assays were preformed on three independent occasions. Results are expressed as the mean ± SE. The survival curves (Fig. 1) were analysed by the Kaplan–Meier method utilizing Graphpad Prism version 5 software. A single variance anova test was carried out on the haemocyte density (Fig. 2) and fungal load (Fig. 3) data using GraphPad Prism version 5.0 Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Survival of G. mellonella larvae following inoculation with glucan prior to C. albicans infection

In order to determine whether different doses of β-glucan would induce a protective effect, larvae of *G. mellonella* were inoculated with various concentrations of β-glucan and incubated for 24 h at 30 °C prior to receiving an inoculum of 1×10^6 *C. albicans* cells. The results (Fig. 1) demonstrate that larvae that had not received a glucan inoculation showed a survival rate of 46.6 ± 7.2%, 33.3 ± 7.1% and 26 ± 7.2% at 24, 48 and 72 h respectively. Larvae that received glucan inoculations of 0.93 or 1.87 μg/larva showed increased survival while larvae inoculated with doses of 7.5, 15, 30 or 60 μg glucan/larva showed survival rates of 100% from 24 to 72 h ($p < 0.05$). This work demonstrates a strong relationship exists between the dose of glucan administered to larvae and the subsequent survival rate following inoculation with a lethal dose of *C. albicans*.

3.2. Administration of glucan leads to alterations in haemocyte density and fungal load in G. mellonella larvae

Fluctuations in haemocyte densities in insects have previously been used as an indicator of the immune response to microbial pathogens [4] and elevated numbers of haemocytes have been associated with a protective immune response against microbial pathogens [18]. In this work the haemocyte density of larvae that had been inoculated with different concentrations of glucan was ascertained at 24 and 48 h. The results (Fig. 2) indicate that after 24 h the haemocyte density of larvae that received a dose of 30 or 60 μg/larva had increased to approximately 75×10^5 per larva ($p < 0.05$). In contrast, those larvae that received a dose of 0.93 μg glucan/larva demonstrated a haemocyte density of $36 \pm 7.9 \times 10^5$ haemocytes per larva which is similar to that in larvae inoculated with PBS ($41 \pm 3.7 \times 10^5$ per larva). In addition those larvae that received a glucan inoculation of 15, 30 or 60 μg/larva had significantly greater haemocyte numbers compared to those larvae that received the lower doses of glucan (0.93, 1.87 or 3.75) at $p < 0.05$. Forty eight hours after administration of glucan the haemocyte density in larvae that received doses of 15, 30 or 60 μg glucan/larva remained elevated ($p < 0.05$) but the haemocyte levels relative to those evident at 24 h had decreased in all glucan-treated larvae possibly indicating a return to 'normal' (pre-inoculation) of haemocyte densities.

The fungal load (i.e. the number of *C. albicans* cells per larva) of larvae pre-inoculated with glucan was also examined. These results demonstrate that following an initial inoculum of 1×10^6 *C. albicans* cells per larva the microbial load increased to $2.4 \pm 0.16 \times 10^6$ per larva in 24 h in those larvae that had received a PBS injection 24 h prior to infection. In contrast those larvae that received a pre-inoculum of 30 or 15 μg glucan/larva demonstrated fungal loads of $4.6 \pm 0.14 \times 10^5$ and $7.2 \pm 0.13 \times 10^5$ *C. albicans* cells per larva respectively at 24 h ($p < 0.05$ relative to the PBS treated larvae). By 48 h the fungal load of those larvae that received glucan inoculations of 1.6–60 μg glucan/larva were all less than the initial inoculum of 1×10^6 cells/larva indicating killing of the yeast cells within the insect haemocoel. The fungal load in PBS treated larvae continued to rise reaching a level of $3.0 \pm 0.62 \times 10^6$ *C. albicans* per larva at 48 h. The fungal loads of larvae that received the higher doses of glucan (15, 30 and 60 μg/larva) were significantly lower than those larvae that received the lower doses of glucan (0.93 and 1.87 μg/larva) at $p < 0.05$ and both time points. These results indicate that glucan administration protects *Galleria* larvae from a subsequent infection with *C. albicans* and leads to a proportional increase in haemocyte density and a concomitant decrease in the fungal load.

3.3. Examination of changes in protein expression following glucan administration

Two-dimensional electrophoresis of haemolymph proteins has been utilised to analyse the immune response of a variety of insects including *Drosophila* [19–21] and *Bombyx mori* [22]. Changes in the expression of selected antimicrobial peptides and proteins of the immune system following exposure to sub-lethal doses of pathogenic fungi have previously been shown to assist in withstanding a subsequent lethal infection and have been visualised by 2D electrophoresis [15]. In the work presented here larvae were inoculated with one of four separate doses of glucan which represented doses that induced no immune priming (i.e. no significant increase in survival following *C. albicans* infection i.e. 0.93 μg glucan/larva), a dose that induced a low level of immune priming (increased survival following *C. albicans* infection i.e. 3.75 μg glucan/larva) and doses that induced a high level of immune priming (increased larval survival, high haemocyte density and reduced fungal load, i.e. 30 and 60 μg glucan/larva) respectively. Haemolymph was extracted after 24 h incubation and proteins were resolved by 2D electrophoresis. The expression

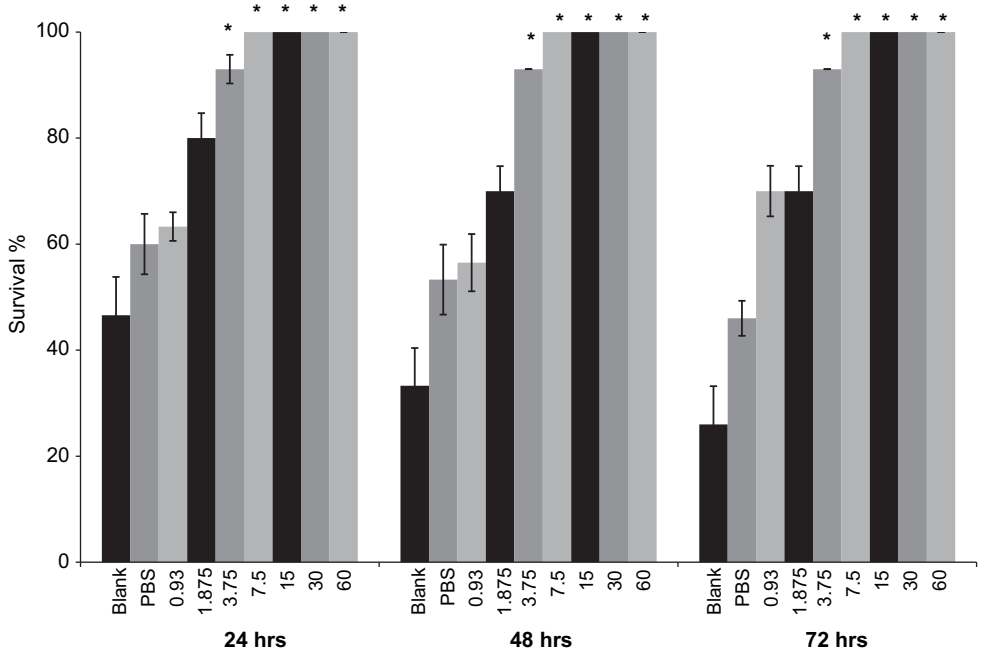

Fig. 1. Survival of *G. mellonella* larvae following inoculation with *C. albicans* after exposure to different doses of β-glucan. Larvae were inoculated with 20 μl of different doses of glucan as indicated 24 h prior to infection with 1×10^6 *C. albicans* cells. Survival was monitored over 72 h. A significant increase in survival relative to that evident in the PBS treated larvae, as determined by the Kaplan–Meier test at $p < 0.05$, is indicated by *.

of selected peptides was analysed and the changes in expression relative to control larvae was plotted against the dose of glucan administered.

The intensity of peptide spots is very strong in the gels of haemolymph from larvae that received the 30 or 60 μg glucan per larva inoculations (Fig. 4). In particular there is a dramatic increase in spot intensity in the pH range 5–7 and size range 45–66.2 kDa. The results presented here demonstrate a gradual increase in the intensity of peptide spots in larvae that had received the glucan inoculations with the greatest increase being evident in those larvae that received the highest glucan doses.

A number of peptides were identified and their expression relative to that in the PBS inoculated larvae was assessed

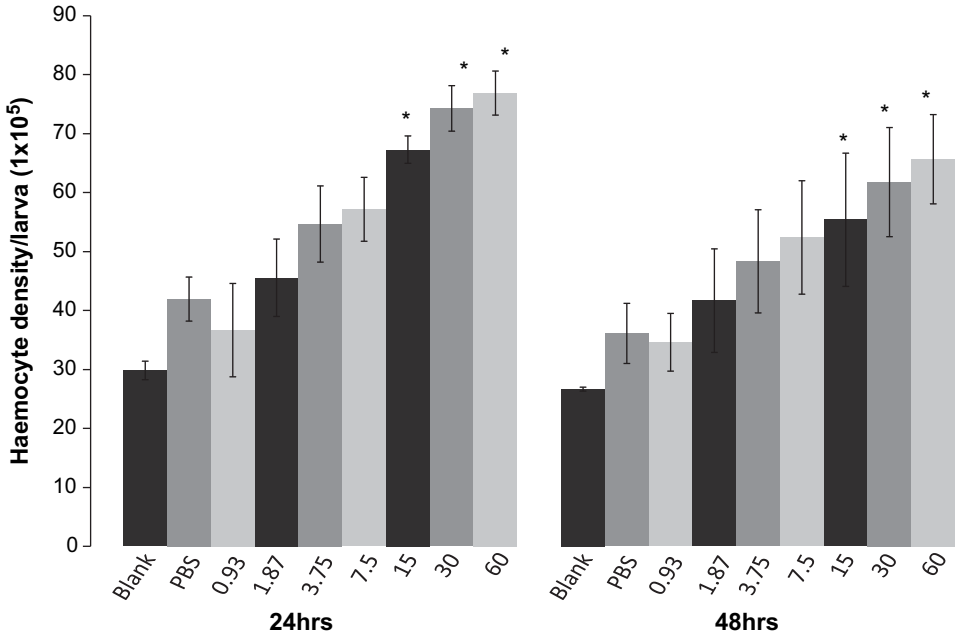

Fig. 2. Haemocyte density in larvae 24 and 48 h after administration of β-glucan. Larvae were inoculated with 20 μl of glucan as indicated and the density of circulating haemocytes was ascertained after 24 and 48 h. A significant increase in haemocyte density relative to that in the PBS treated larvae at $p < 0.05$ is indicated by *.

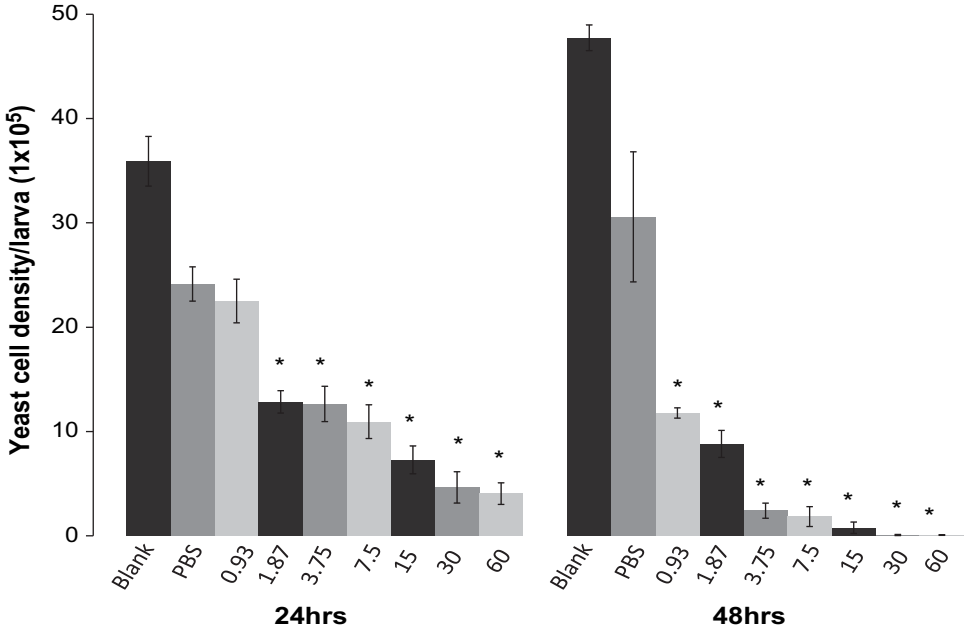

Fig. 3. Fungal loads in larvae challenged with *C. albicans* following administration of β-glucan. Larvae were inoculated with 20 μl of β-glucan 24 h prior to infection with 1×10^6 *C. albicans*. The fungal load was ascertained by serially diluting homogenized larvae and plating aliquots onto erythromycin containing agar plates. A significant change in fungal load per larva relative to that in the PBS treated larvae at $p < 0.05$ is indicated by *.

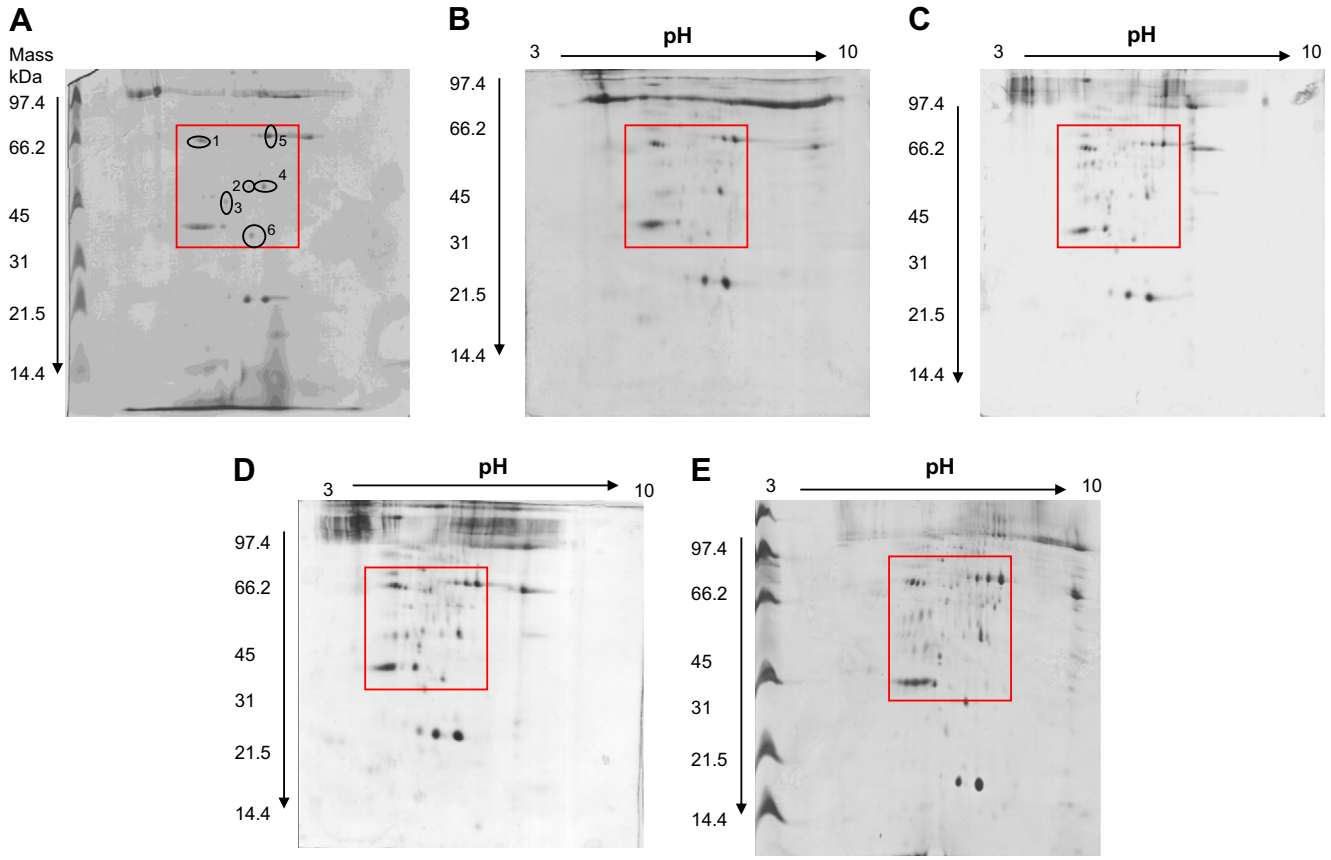

Fig. 4. Two-dimensional SDS-PAGE of haemolymph from larvae challenged with glucan. Larvae were inoculated with increasing doses of glucan and the proteomic profile prepared as described. A: Profile of haemolymph from larvae inoculated with PBS, Haemolymph profile of larvae inoculated with B: 0.9375 μg glucan/larva, C: 3.74 μg/larva, D: 30 μg/larva or E: 60 μg/larva. The area in each gel marked with the box indicates an area of high variation in peptide intensity.

densitometrically. The relative expression of putative tryptophanyl-tRNA synthetase (*z* score 1.44, % coverage 16) demonstrated a clear increase with increasing doses of glucan and showed a three-fold increase in expression in larvae that received a 60 μg glucan per larva (Fig. 5). The intensity of putative ATP synthase (*z* score 1.89, % coverage 8) remained constant in larvae that had received the lower concentrations of glucan but rose 1.43 and 2.04 fold in those larvae inoculated with 30 and 60 μg glucan per larva, respectively (Fig. 5). A dose of 3.75 μg glucan per larva was sufficient to cause an increase in the expression of the insect immune protein hemolin (*z* score 1.88, % coverage 22). In contrast the relative expression of the putative antimicrobial peptide archaemetzincin (*z* score 2.11, % coverage 23) falls upon administration of 0.93, 3.75 and 30 μg glucan per larva relative to the control and only shows an increase in intensity following administration of 60 μg glucan per larva. The putative cathelin-related antimicrobial peptide (*z* score 1.98, % coverage 28) showed increased expression following administration of 30 and 60 μg glucan per larva.

4. Discussion

The data presented here demonstrate that administration of glucan primes the immune response of *G. mellonella* larvae and enables them to withstand a subsequent infection with a lethal dose of *C. albicans*. The ability of an insect to increase its antimicrobial defences following challenge with a low level of pathogen has advantages as it may protect the insect from a subsequent potentially larger infection [23] but can be fatal if compensatory feeding is unavailable [24]. Previous work has demonstrated that fungal cell wall components (e.g. mannan, laminarin) or sub-lethal doses of yeast cells have a similar immune priming effect on inoculated *G. mellonella* larvae [15]. However the work presented here demonstrates for the first time a correlation between the amount of glucan administered and the degree of immune priming evident in larvae. Glucan doses of 15, 30 or 60 μg glucan per larva were sufficient to render the larvae resistant to *C. albicans* infection and provoked the greatest increase in haemocyte density and largest reduction in fungal loads at 24 and 48 h. In contrast larvae that received a glucan dose of 0.93 μg glucan per larva showed little increase in survival relative to that seen in PBS inoculated larvae. In this case there was no significant increase in the haemocyte density or decrease in the fungal load. These results demonstrate that while glucan administration does prime the insect immune response the extent of this increase appears to be proportional to the size of the glucan inoculum.

Analysis of the proteome of larvae inoculated with glucan demonstrated peptides of greater intensity in the haemolymph from larvae that received the highest glucan doses (i.e. 30 and 60 μg glucan per larva). Examination of selected peptides revealed that the expression of all peptides was not elevated to the same extent following administration of glucan. For example, the expression of putative insect immune protein hemolin increased dramatically following administration of 3.75 μg glucan per larva while that of putative archaemetzincin only increased following inoculation of larvae

Protein name	β Glucan Concentration μg/20μl					Change in fold expression				
	0	0.9375	3.75	30	60	0	0.9375	3.75	30	60
1. Insect immune protein hemolin						1	1.04	1.96	1.95	2.18
2. Archaemetzincin						1	-0.072	0.18	0.93	2.89
3. Tryptophanyl-tRNA synthetase						1	1.11	1.63	2.01	3.19
4. Cathelin-related antimicrobial peptide						1	0.018	0.79	1.31	2.02
5. ATP synthase						1	1	0.96	1.43	2.04
6. Loading Control (storage protein)						1	0.96	0.98	1.06	1.02

Fig. 5. Relative expression of selected peptides in glucan inoculated larvae. The relative intensity of selected peptide spots visible on two-dimensional SDS-PAGE gels. The fold change in expression was ascertained following densitometric analysis. The expression of the putative peptides in the haemolymph of control larvae is recoded as 1. Spot numbers 1–6 refer to spots marked in Fig. 4A.

with a dose of 60 μg glucan per larva. This demonstrates that while administration of glucan leads to increased expression of antimicrobial peptides and immune peptides the size of the dose determines which peptides will be elevated in expression.

Immune priming in insects by exposure to sub-lethal doses of yeast, fungal cell wall components [15], alterations in temperature [25] or physical stress [26] has been shown to be mediated by alterations in the humoral and cellular immune responses. Immune priming has a survival advantage for insects as it enables the up-regulation of immune protection following a low level insult and in advance of a potentially lethal subsequent insult but it does impose a cost on the insect and, in the absence of compensatory feeding, can be fatal [24]. While the work presented here demonstrates that administration of glucan induces immune priming it is clear that the amount of glucan administered to the insect determines the degree of the immune response.

The ability to differentiate between different levels of glucan may allow the insect balance the amount of resources that must be committed to elevating the immune response against the likely threat posed by the presence of the foreign material within the haemolymph. So, for example, a low dose of glucan could lead to small changes in the haemocyte density and the increased expression of some but not all antimicrobial peptides while a large dose of glucan triggers large increases in haemocyte density and elevated expression of a wide range of immune proteins and antimicrobial peptides. This result indicates that priming the immune system with glucan does increase protein expression and the size of the inoculum will determine what proteins will be increased in expression. Entry of a low level of pathogen or pathogen associated material would appear to activate a response designed to eliminate this threat. However there would appear to be a threshold above which a more comprehensive immune response is initiated with the potential to eliminate the foreign agent and withstand the entry of a second potentially lethal inoculum 24–48 h later. Below a certain threshold the immune response is aimed at curtailing the infection but above this the immune response is aimed at protecting the insect from subsequent infection as well as curtailing and/or eliminating the immune priming agent (microbial cell or cell wall material).

The data presented here further strengthen the view that insects can modulate their immune responses depending upon the nature and size of the microbial challenge. The appearance of a super-spreading haemocyte in *Manduca sexta* was seen after microbial challenge but not following wounding [27] indicating a differential response to a microbial challenge compared to a physical insult. Recent work has also highlighted the activation of a number of genes following administration of fungal cell wall material to *G. mellonella* larvae [15] however only a subset of genes were activated when insects were physically challenged although both treatments lead to immune priming [26]. It has also been established that immune stimulation of honey bees, *Apis mellifera,* by inoculation with lipopolysaccharide alters the expression of genes coding for defensin and alters the behavior of challenged bees [28].

While it is well established that insects can discriminate between different challenges [3,6,9–11,14] the work presented here has demonstrated that they can also differentiate between different doses of the same challenge (e.g. glucan) and respond accordingly. This ability would allow insects to fine tune their immune response and ensure adequate protection against an invading pathogen or injury while minimizing the amount of resources that must be directed towards the immune response. This strategy would have strong survival advantages as it would allow insects withstand infection following an initial priming event without the use of excess resources if the challenge was limited in nature or could be neutralized easily [24]. The results presented here, together with those of others [27], are beginning to unravel the complexity of the insect immune response and its ability to selectively respond to a variety of challenges in order to ensure survival.

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