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Original article

Disruption of haemocyte function by exposure to cytochalasin b or nocodazole increases the susceptibility of *Galleria mellonella* larvae to infection

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Abstract

Administration of non-toxic concentrations (10 μM) of cytochalasin b and nocodazole to larvae of *Galleria mellonella* increased their susceptibility to infection by the yeast *Candida albicans*. These agents were found to inhibit the process of phagocytosis and to reduce the killing ability of haemocytes. In addition, both cytochalasin b and nocodazole reduced the release of antimicrobial peptides (e.g. apolipophorin 3) and enzymes (e.g. serine protease) from PMA stimulated haemocytes. Rhodamine coupled phalloidin staining revealed reduced F-actin formation in haemocytes treated with nocodazole or cytochalasin b. By disrupting the formation of F-actin cytochalasin b and nocodazole have the ability to retard the function of haemocytes, in the same manner as they affect mammalian neutrophils, and thus increase the susceptibility of larvae to infection. The results presented here demonstrate that haemocytes are sensitive to inhibition by nocodazole and cytochalasin b, in a similar manner to neutrophils, thus highlighting another similarity between both cell types and so increasing the attractiveness of using insects as alternative models to the use of mammals for *in vivo* pathogen or drug screening.

Keywords: Cytochalasin b; *Galleria*; Haemocyte; Mini-host; Nocodazole; Neutrophil

1. Introduction

The immune response of insects bears a number of structural and functional similarities to the innate immune response of mammals [1] and as a consequence insects may be used to predict the likely response of mammals to a variety of bacterial [2] and fungal [3,4] pathogens. At the cellular level the immune cells of insects (haemocytes) and mammals (neutrophils) demonstrate a number of similarities in terms of their abilities to phagocytose and kill microbial pathogens [5].

Phagocytic haemocytes (plasmatocytes and granulocytes) play a central role in the insect immune response and function in a similar manner to mammalian neutrophils by phagocytosing and destroying invading microorganisms [6]. The burst in oxidative metabolism associated with activation of either human neutrophils or insect haemocytes results in the production of reactive oxygen species (ROS) with evidence of increased oxygen consumption resulting in superoxide (O_2^-) production by haemocytes of *Galleria mellonella* [5]. A number of cytosolic proteins ($p67^{phox}$, $p47^{phox}$ $p40^{phox}$ and $p21^{rac}$) are required to activate the membrane bound flavocytochrome b_{558} of the NADPH-oxidase of neutrophils and these translocate to the phagocytic vacuole membrane upon activation. Using immunological and matrix-assisted laser desorption ionisation-time of flight analysis (MALDI-TOF), the presence of homologous proteins to $p67^{phox}$ and $p47^{phox}$ was found in haemocytes of *G. mellonella* [5]. In addition these proteins translocate from the cytosol to the membrane fraction upon PMA stimulation in a similar manner to the equivalent proteins in human neutrophils [7].

Abbreviations: DMSO, dimethy sulphoxide; IPS, insect physiological saline; PMA, phorbol-12-myristate-13-acetate.

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Neutrophil mediated microbial killing also requires the release of antimicrobial peptides and proteins from cytosolic granules. These granules migrate through the cytosol, whereupon the granule membrane fuses with the invaginated cell membrane, followed by release of the granule contents into the phagocytic vacuole and around the exterior of the cell in the process of degranulation. The released enzymes attack the cell wall of microbes that are too large to ingest and also play a vital role in killing microbes that have been phagocytosed. F-actin formation plays a central role in exocytosis of granules and the inhibition of this process by compounds such as cytochalasin b and nocodazole reduces neutrophil mediated exocytosis [8,9]. Cytochalasin b has been shown to weaken actin filaments so reducing the neutrophil's ability to produce pseudopodia and phagocytose [9,10]. Nocodazole inhibits the uptake of particles and retards phagocytosis by diminishing α tubulin polymerisation [10,11]. Cytochalasin b and nocodazole are capable of microfilament-disruption and microtubule depolymerization, respectively, and these changes result in a severe effect on cells where actin filaments are shortened and weakened [12].

The aim of the work presented here was to assess the effect of cytochalasin b and nocodazole on the susceptibility of *G. mellonella* larvae to infection and to establish whether these agents affected the function of haemocytes in a similar manner to the manner in which they disrupt the action of neutrophils.

2. Material and methods

2.1. Chemicals

All chemicals and reagents were of the highest purity and were purchased from Sigma Aldrich Chemical Co. Ltd., Dorset, United Kingdom, unless otherwise stated. Cytochalasin b and nocodazole were dissolved in DMSO and diluted to 10 μM which had a corresponding concentration of 0.001% (v/v) DMSO. Relevant solvent controls were employed in all assays.

2.2. Microbial strains and culture conditions

Candida albicans MEN (a kind gift from Prof. D. Kerridge, Cambridge, UK) was cultured in YEPD broth (2% (w/v) glucose, 2% (w/v) Bactopeptone, 1% (w/v) yeast extract (Oxoid Ltd., Basingstoke, England)) at 30 °C in an orbital shaker. *Aspergillus flavus* was grown on malt extract agar plates and conidia were harvested by washing with PBS-Tween (0.01% v/v) solution.

2.3. Administration of cytochalasin b and nocodazole to G. mellonella

Larvae (approximately 0.3 g in weight, 2 weeks old) were inoculated through the last left pro-leg into the haemocoel with 20 μl (10 μM cytochalasin b and/or 10 μM nocodazole) using a myjector syringe (Thermo Europe, Leuven, Belgium) and placed in the dark for 4 h at 30 °C. All *in vivo* experiments included controls which consisted of larvae injected with sterile PBS (20 μl) or 0.001% (v/v) DMSO. Larvae were subsequently injected with *C. albicans* cells (1×10^6 in 20 μL) and incubated at 30 °C. Administration of these agents to the larvae did not result in any decrease in viability even after 72 h incubation (data not presented).

2.4. Isolation of insect haemocytes

Haemocytes were extracted from sixth-instar larvae of *G. mellonella* by bleeding serum from 10 larvae into 10 ml of sterile Insect Physiological Solution (IPS; 150 mM NaCl, 5 mM KCl, 0.1 M Tris–HCl, 10 mM EDTA and 30 mM Trisodium citrate in dH_2O, pH 6.9). Cells were harvested by centrifugation at 1000× *g*, washed once and finally resuspended in 5 mM PBS-Glucose containing 1 mg/ml Pepstatin A, 1 mg/ml Aprotitin, 1 mM PMSF and 1 mg/ml Leupeptin. Haemocyte viability was assessed by trypan blue exclusion [13].

2.5. In vitro killing of C. albicans by insect haemocytes

Yeast cells were chosen as a target organism in order to measure fluctuations in the fungicidal abilities of nocodazole or cytochalasin b treated haemocytes. *C. albicans* cells (2×10^5) were opsonised using cell free haemolymph diluted in IPS for an incubation period of 30 min at 37 °C. Killing of yeast cells was measured by incubating 2×10^5 yeast cells with 1×10^5 haemocytes in a stirred chamber at 37 °C. A 200 μl aliquot was removed immediately after addition of the yeast cells (time zero) and after 20, 40, 60 and 80 min, diluted 1 in 5 in Minimum Essential Medium (Sigma Aldrich) prior to plating onto YEPD plates supplemented with erythromycin to prevent bacterial growth. The viability of yeast cells was assessed by determining colony number and this experiment was performed on three separate occasions. The viability of the haemocytes at time zero was 87.8 ± 2.7% and after 80 min incubation viability was 81.7 ± 8.1% ($p = 0.536$).

Haemocytes (1×10^5) were exposed to cytochalasin b (10 μM) or nocodazole (10 μM) for 30 min at 37 °C prior to addition to the opsonised *C. albicans*. The viability of haemocytes prior to exposure to these agents was 87.8 ± 2.7%. After 30 min haemocyte viability was 87.0 ± 2.7% and 90.0 ± 2.8% in those cells exposed to cytochalasin b and nocodazole, respectively.

2.6. Assessment of phagocytic ability of haemocytes

Haemocytes (5×10^6/ml, viability 87.8 ± 1.3%) were isolated and incubated in the presence of cytochalasin b (10 μM) and/or nocodazole (10 μM) for 30 min at 37 °C. Viability of cells after incubation with cytochalasin b was 75.7 ± 4.7% and 82.9 ± 7.3% after incubation with nocodazole. Phagocytosis was measured by incubating pre-treated haemocytes (5×10^6/ml) with opsonised *A. flavus* conidia (1×10^7) in a final volume of 1 ml. This was stirred in a thermally controlled chamber at 37 °C. An aliquot was removed at 30 min and at 90 min. Conidia of *A. flavus* were chosen for this experiment as their colour (dark

green) made it easier to visualize when they had been internalised by the haemocytes. One hundred haemocytes were examined microscopically and the number containing phagocytosed conidia was ascertained on three independent occasions. The mean number ± standard error was calculated.

2.7. 2-D electrophoresis and mass spectrometry

Haemocytes (1×10^8) were PMA (Phorbol-12-myristate-13-acetate concentration of PMA) stimulated and the released proteins were acetone precipitated and resuspended in IEF buffer (8 M Urea, 2 M Thiourea, 4% (wt/vol) CHAPS, 1% (vol/vol) Triton X-100, 65 mM DTT, 10 mM Trizma Base and 0.8% (vol/vol) Ampholytes (Amersham Bioscience, United Kingdom)). The protein content was quantified by Bradford Assay and the protein solution was brought to a 250 μL volume for 13 cm IPG strips with IEF buffer. Strips were allowed to rehydrate and protein (300 μg) was loaded onto the 13 cm strips. Resolution in the first and second dimensions was performed as described [14]. Mass spectrometric analysis of trypsin digested proteins was performed using an Agilent ESI Trap LC/MS. Resulting spectra were analysed using MASCOT, Matrix Sciences with a score over 80 considered significant (www.matrixscience.com/search).

2.8. Confocal immunofluoresence microscopy

Cells were also prepared for rhodamine phalloidin (Biotium, Inc.) staining which is specific for F-actin. Cells were fixed and permeabilised as described above. Cells were washed with PBS, 10 μL of methanol stock solution of the rhodamine phalloidin was diluted in 200 μL PBS with 1% BSA. A sample of this (50 μL) was added to the 1×10^4 fixed and permeabilised haemocytes on each slide. This was added for 20 min at room temperature. The cells were washed 2–3 times with PBS prior to viewing. All slides were sealed and viewed with an Olympus Flouview 1000 confocal microscope, and the data analysis was completed by utilizing the Olympus Fluoview 1000 software package for 3 independent cells for each treatment. A 20 μM diameter was drawn through each cell and variations in the fluorescence along this line were analysed.

2.9. Statistical analysis

All assays were performed on three independent occasions and results are expressed as the mean ± SE. Statistical comparisons were made with Students *t* test. The survival data were analysed by the Kaplan–Meier method utilizing Graphpad Prism version 5 software. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Effect of cytochalasin b and nocodazole on the susceptibility of G. mellonella larvae to infection

Administration of a non-toxic concentration of cytochalasin b or nocodazole (10 μM) to larvae of *G. mellonella* 4 h in advance of a potentially lethal dose of *C. albicans* (5×10^6 cells/20 μL) resulted in increased susceptibility of the treated larvae to infection (Fig. 1). Larvae that received nocodazole showed approximately 10% survival at 24 h compared to the control larvae 26.6% ($p < 0.05$) and those larvae that received cytochalasin b showed approximately 6.6% survival. Larvae that received a mixture of nocodazole and cytochalasin b showed survival of approximately 3.33%. Untreated larvae or those that received DMSO (0.001%) displayed survival rates of approximately 26.6% at 24 h. These results demonstrate that nocodazole and cytochalasin b can impair the immune function of larvae and increase susceptibility of larvae to infection by *C. albicans*.

3.2. Effect of nocodazole and cytochalasin b on haemocyte mediated phagocytosis and killing

Cytochalasin b and nocodazole have previously been shown to inhibit the uptake of particles during neutrophil mediated phagocytosis [15]. In order to evaluate the effect of cytochalasin b and nocodazole on the ability of haemocytes to phagocytose pathogens, haemocytes were extracted and exposed to cytochalasin b and/or nocodazole for 30 min prior to co-incubation with conidia of *A. flavus*. The rate of internalization of conidia was measured as described. The results demonstrate that 61.3% of DMSO treated haemocytes and 55.0% of untreated haemocytes had engulfed conidia at 90 min (Fig. 2). In contrast those haemocytes that had been pre-exposed to cytochalasin b or nocodazole showed 21.3% ($p = 0.013$) and 21.0% ($p = 0.02$) phagocytosis. Exposure of haemocytes to both agents resulted in a phagocytosis rate of 31.7% ($p = 0.033$).

The effect of nocodazole and/or cytochalasin b on the ability of haemocytes to kill ingested microbial cells was measured as described. The results indicate that haemocytes can kill yeast cells but that in the presence of the inhibitors cytochalasin b or nocodazole, alone or in combination, this process is retarded or inhibited (Fig. 3). For example, 62.67 ± 7.7% of yeast cells were killed by haemocytes at 40 min while haemocytes treated with cytochalasin b killed only 7.67 ± 7.09% of cells and 19.5 ± 29 were killed by haemocytes that had been exposed to nocodazole at the same time point ($p < 0.05$). At 80 min 84.6 ± 11.1% of yeast cells were killed by control haemocytes, while cytochalasin b treated haemocytes had killed 49 ± 9.2% and nocodazole treated haemocytes had killed 47.5 ± 13.8% yeast cells ($p < 0.05$). Haemocytes treated with a combination of the two inhibitors were incapable of killing yeast cells.

These results indicate that prior exposure of haemocytes to cytochalasin b, nocodazole or a combination of both agents significantly reduces the phagocytic and microbicidal abilities of haemocytes without significantly affecting haemocyte viability.

3.3. Analysis of the effect of nocodazole and cytochalasin b on protein release from PMA stimulated haemocytes

The process of degranulation in neutrophils involves the release of a range of enzymes into the phagocytic vacuole and

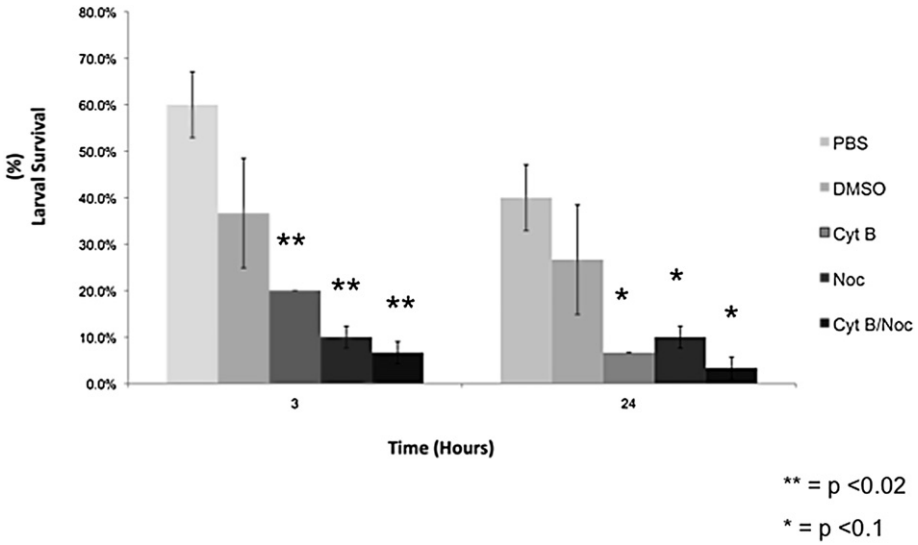

Fig. 1. Susceptibility of larvae to *C. albicans* infection. Larvae were administered non-toxic concentrations of cytochalasin b or nocodazole 4 h in advance of infection with *C. albicans*.

around the cell periphery [16]. Cytochalasin b has previously been shown to inhibit the process of degranulation in neutrophils [9]. The release of intracellular components from the cell can be visualized by stimulating cells with PMA and isolating the released material in the secretome. The work described here sought to establish whether cytochalasin b and nocodazole could inhibit the release of proteins from haemocytes in a similar manner to their effects on neutrophils. Haemocytes from *G. mellonella* were exposed to nocodazole or cytochalasin b as described. Cells were subsequently stimulated with PMA in a stirred chamber at 37 °C for 6 min. The secreted proteins were precipitated and resolved by 2-D electrophoresis. Selected peptides showing alteration in expression were excised, digested and identified using LC-Mass spectrometry. The relative abundance of secreted proteins was quantified on the basis that the amount released by the PMA treated haemocytes was 100%.

One peptide (Spot 1) (Figs. 4 and 5) was identified as showing homology to Apolipophorin 3 (54% sequence coverage, mascot score 299) which plays an essential role in modulating the immune response in *G. mellonella* [17]. It is located in the cytoplasm of haemocytes and also on the surface of granules [17,18]. Exposure of haemocytes to nocodazole and cytochalasin b reduced the release of apolipophorin 3 from haemocytes to 37% and 40%, respectively, relative to that seen in PMA stimulated haemocytes. Another peptide

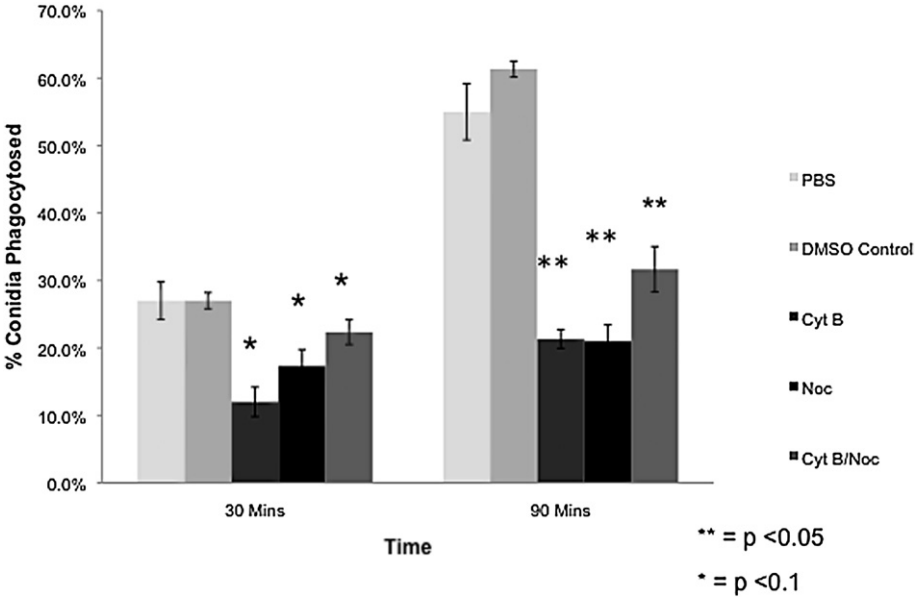

Fig. 2. Effect of cytochalasin b and nocodazole on phagocytosis ability of haemocytes. Isolated haemocytes were treated with cytochalasin b or nocodazole and then incubated in the presence of opsonised conidia of *A. flavus*. The rate of phagocytosis was assessed by microscopic examination.

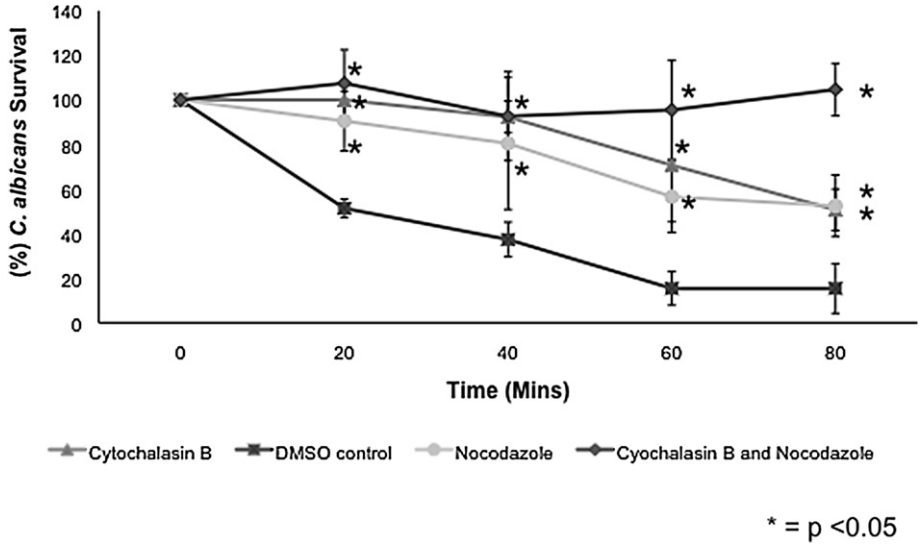

Fig. 3. Effect of nocodazole and cytochalasin b on ability of G. mellonella haemocytes to kill *C. albicans* cells. Opsonised *C. albicans* cells were incubated with haemocytes and the viability of yeast cells was monitored by plating aliquots of yeast suspension onto agar plates. Haemocytes were exposed to the inhibitors prior to co-incubation with the yeast cells. $p < 0.005$ (**), $p < 0.05$ (*) relative to % survival in DMSO treated control.

(Spot 2) showed homology to ferritin (39% sequence coverage, mascot score 554). Apoferritin exists in the haemocytes of insects [19]. In this case haemocytes treated with nocodazole showed a 45% reduction in the release of this protein while those treated with cytochalasin b demonstrated a 33% inhibition. Spot 3 showed homology to serine protease (mascot score of 105 and 8% sequence coverage). When haemocytes were treated with cytochalasin b there was a 22% decrease in release of serine protease, with the addition of nocodazole there was a 10% decrease in release of this protein. It has previously been demonstrated that the inhibition of serine proteases abolished the killing potential of neutrophils [20]. Spot 4 was identified as a putative transferrin precursor with 5% coverage and a mascot score of 159. Transferrin

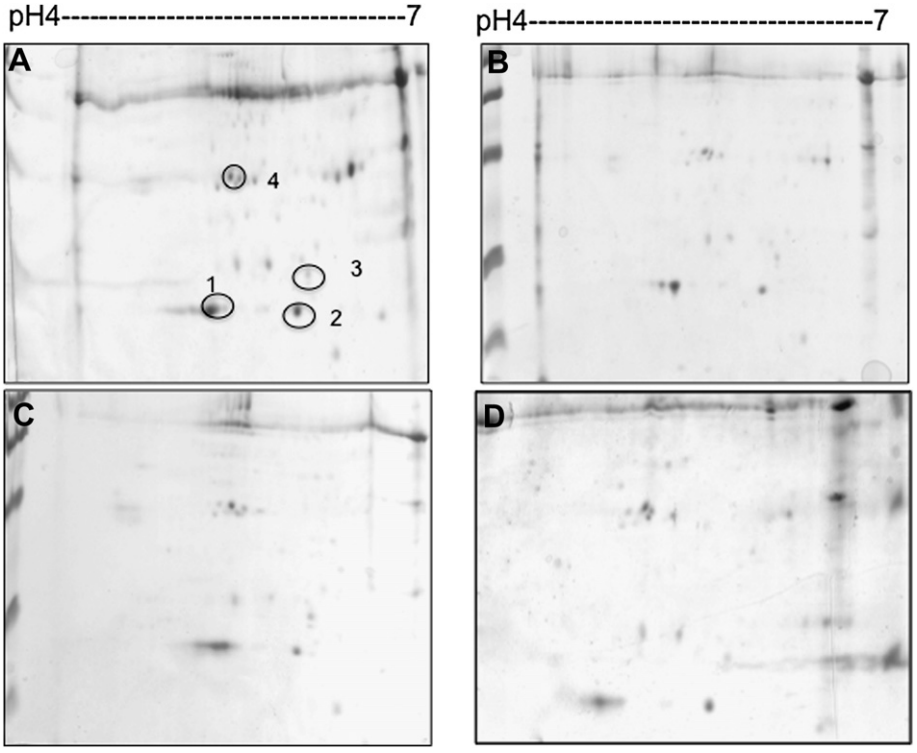

Fig. 4. Two-dimensional SDS-PAGE of peptides released from *G. mellonella* haemocytes. Haemocytes were induced to degranulate as described and the secretome was isolated and resolved by 2D electrophoresis. (A) PMA stimulated, (B) unstimulated, (C) cytochalasin b + PMA, (D) nocodazole + PMA.

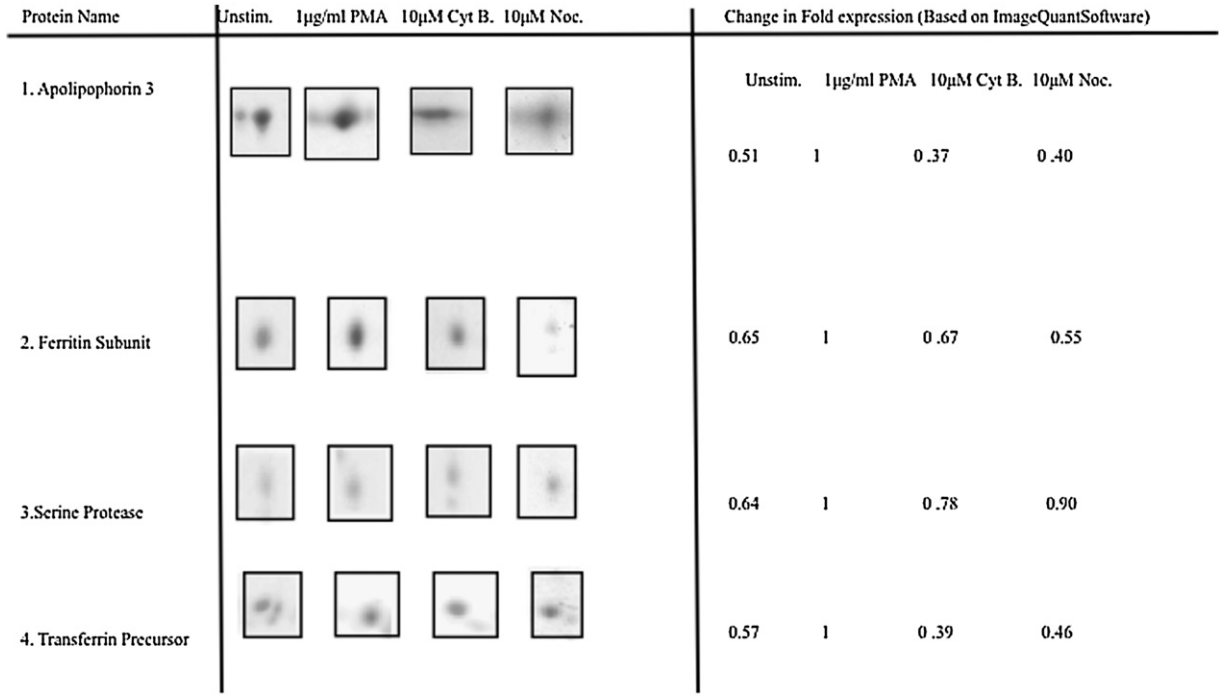

Fig. 5. Relative expression of selected peptides released by *G. mellonella* haemocytes. The relative intensity of selected peptide spots visible on two-dimensional SDS-PAGE gels was determined. Spot numbers 1–4 refer to spots marked in Fig. 4A.

precursor is up-regulated in the haemocyte of the mosquito *Culex quinquefasciatus* upon infection [21]. Transferrin release is reduced in nocodazole and cytochalasin b treated haemocytes by approximately 60%. These results indicate a reduction in the release of proteins that are normally released during the process of degranulation thus indicting that the two agents retard the degranulation process.

3.4. Visualisation of the effect of nocodazole and cytochalasin b on F-actin formation in haemocytes

Actin exists in the G form in unstimulated neutrophils and is converted to the F form in order to perform the structural and morphological changes associated with activation (e.g. phagocytosis, degranulation) [22]. Rhodamine phalloidin staining was used to visualize the formation and distribution of F-actin within haemocytes. Using this stain it can be seen that PMA stimulated haemocytes have high levels of F-actin while the control cells show weak fluorescence indicating that most of the actin is still in the G form. Haemocytes treated with nocodazole or cytochalasin b in advance of PMA stimulation show levels of fluorescence similar to that in unstimulated cells indicating that the formation of F-actin is reduced (Fig. 6). This can be visualized in the graph of relative fluorescence intensity along 20 µm diameters of cells (Fig. 6B). PMA treated cells show the greatest fluorescence while unstimulated and inhibitor treated cells show reduced fluorescence.

4. Discussion

The insect immune response demonstrates a number of similarities to the innate immune response of mammals [1]. Both immune systems contain cellular and humoral responses that can counteract and kill invading microbial pathogens. Haemocytes phagocytose and kill microbes by the generation of superoxide, which is achieved by the NADPH-oxidase complex that is homologous to that in neutrophils [5]. While insects, and *G. mellonella* in particular, are useful and highly effective alternatives to the use of mammals in routine drug [23] and pathogen screening [2–4,24] their utility can only be fully exploited if it is possible to validate their use. This may be achieved by characterizing the similarities between the immune response of mammals and the innate immune response of humans.

Cytochalasin b and nocodazole have well characterized effects on the activity and function of neutrophils. By disrupting F-actin assembly the inhibitors employed here have the ability to adversely affect phagocytosis, NADPH-oxidase formation and degranulation by neutrophils [9]. The objective of the work presented here was to establish whether they had a similar effect on the function of insect haemocytes and so render *G. mellonella* larvae susceptible to infection.

Administration of non-toxic concentrations of cytochalasin b or nocodazole to *G. mellonella* larvae increased their susceptibility to infection with *C. albicans*. Those larvae that received nocodazole 4 h prior to infection with *C. albicans* showed 10% survival at 24 h compared to approximately 36% survival in the control larvae. Larvae administered cytochalasin b in advance of infection showed similar levels of survival at 24 h to the nocodazole treated larvae.

Exposure of haemocytes to cytochalasin b or nocodazole reduced the ability of haemocytes to engulf *A. flavus* conidia. Approximately 60% of control haemocytes had engulfed conidia after 90 min compared to 20% of those haemocytes

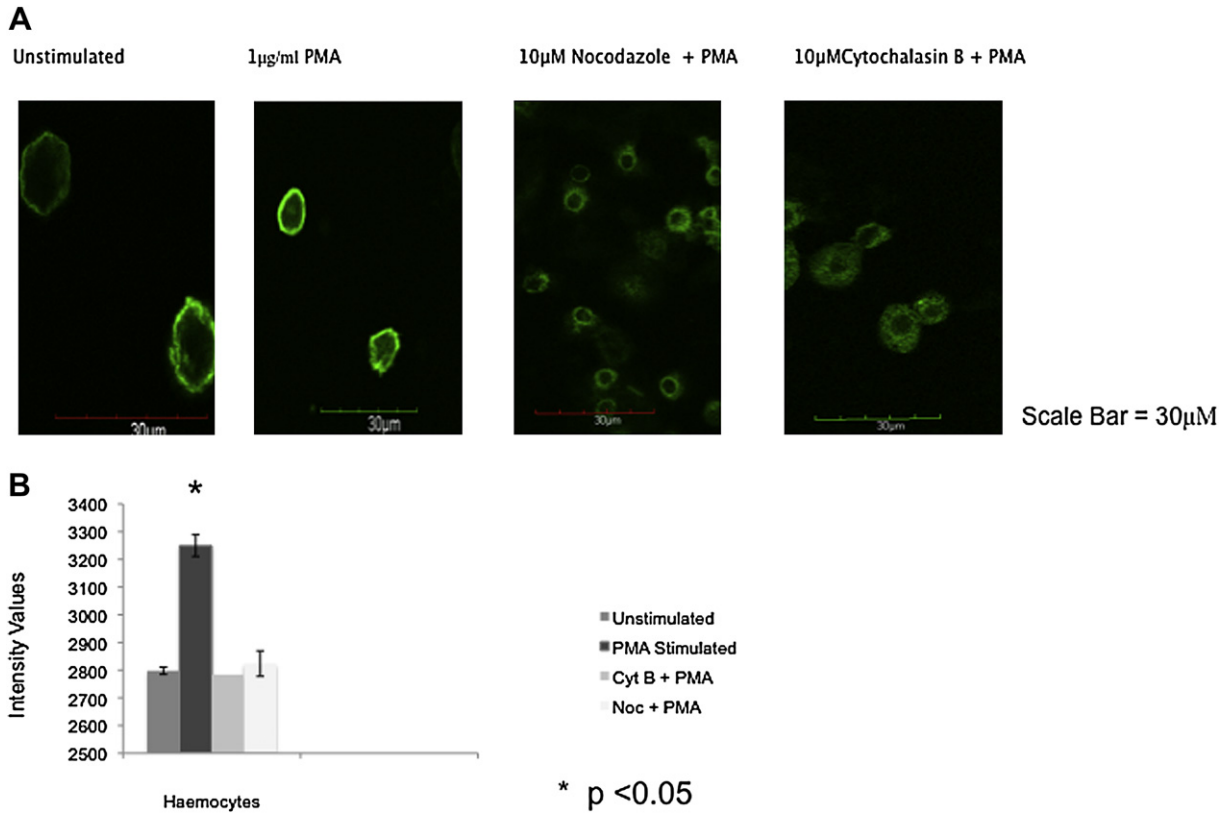

Fig. 6. (A) Distribution of F-actin in *G. mellonella* haemocytes following exposure to nocodazole or cytochalasin b prior to PMA stimulation. Use of rhodamine coupled phalloidin staining to visualize the distribution of F-actin in unstimulated and stimulated haemocytes previously exposed to cytochalasin b or nocodazole. (Magnification × 400). (B) Relative intensity of rhodamine coupled phalloidin staining in cells.

that had been exposed to nocodazole or cytochalasin b. This suggests that, while the concentration used here did not adversely affect the viability of haemocytes, the agents reduce the ability of haemocytes to undergo the morphological changes required to engulf a target. Treatment of haemocytes with cytochalasin b or nocodazole also severely reduced the ability of haemocytes to kill a pathogenic target. Indeed exposure of haemocytes to both inhibitors simultaneously completely abolished their ability to kill without significantly affecting their viability. These results indicates that the ability of the inhibitors to prevent F-actin formation, which is essential for phagocytosis, superoxide production and degranulation in neutrophils [8,9] renders the haemocytes incapable of phagocytosis and killing which may explain the increased susceptibility of larvae to *C. albicans* infection following administration of the agents.

The secretome of haemocytes that had been previously treated with nocodazole or cytochalasin b prior to PMA stimulation was compared with that from untreated haemocytes. The results reveal that the release of a number of proteins known to be associated with haemocytes is reduced following treatment of cells with inhibitors. In particular the release of apolipophorin, ferritin, serine protease and transferrin precursor is reduced. Examination of the rhodamine staining of haemocytes indicated greatest fluorescence in PMA treated cells but reduced level in those that were unstimulated or treated with cytochalasin b or nocodazole. This result indicates a reduction in the rate of F-actin formation.

The data presented here indicate that the use of cytochalasin b and nocodazole can render *G. mellonella* larvae susceptible to infection and that this is mediated by the disruption of haemocyte function but not a reduction in the viability of haemocytes. Haemocytes demonstrate many similarities to neutrophils in terms of their structure and function. In addition, inhibitors of neutrophil function (e.g. gliotoxin) [25] also disrupt the function of insect haemocytes [7] which highlights the similarities between the two cells types. These results establish another similarity between mammalian neutrophils and insect haemocytes and reinforce the validity of using insects as models for studying virulence of human microbial pathogens [4,24,26], and for screening the *in vivo* action of antimicrobial drugs [23].

Acknowledgements

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References

[1] K. Kavanagh, E.P. Reeves, Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens, FEMS Microbiol. Rev. 28 (2004) 101–112.

[2] G. Jander, L. Rahme, F. Ausbel, Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects, J. Bacteriol. 182 (2000) 3843–3845.
[3] M. Brennan, D.Y. Thomas, M. Whiteway, K. Kavanagh, Correlation between virulence of *Candida albicans* mutants in mice and *Galleria mellonella* larvae, FEMS Immuno. Med. Mycol. 34 (2002) 153–157.
[4] B. Fuchs, E. Mylonakis, Using non-mammalian host to study fungal virulence and host defense, Curr. Opin. Microbiol. 9 (2006) 346–351.
[5] D. Bergin, E.P. Reeves, J. Renwick, F.B. Wientjes, K. Kavanagh, Superoxide production in haemocytes of *Galleria mellonella* – identification of proteins homologous to the NADPH oxidase complex of human neutrophils, Infect. Immun. 73 (2005) 4161–4173.
[6] S. Tojo, F. Naganuma, K. Arakawa, S. Yokoo, Involvement of both granular cells and plasmatocytes in phagocytic reactions in the greater wax moth, *Galleria mellonella*, J. Insect Physiol. 46 (2000) 1129–1135.
[7] J. Renwick, E.P. Reeves, F.B. Wientjes, K. Kavanagh, Translocation of proteins homologous to human neutrophil $p47^{phox}$ and $p67^{phox}$ to the cell membrane in activated hemocytes of *Galleria mellonella*, Dev. Comp. Immunol. 31 (2007) 347–359.
[8] J. Bylund, S. Pellmé, F. Huamei, U.H. Mellqvist, K. Hellstrand, A. Karlsson, C. Dalgren, Cytochalasin B triggers a novel pertussis toxin sensitive pathway in TNF-alpha primed neutrophils, BMC Cell Biol. 5 (2004) 5–21.
[9] T. Bengtsson, C. Dahlgren, O. Stendahl, T. Andersson, Actin assembly and regulation of neutrophil function: effects of cytochalasin B and tetracaine on chemotactic peptide-induced O_2-production and degranulation, J. Leuk. Biol. 49 (1991) 236–244.
[10] I. Neeli, N. Dwivedi, S. Khan, M. Rad, Regulation of extracellular chromatin release from neutrophils, J. Innate Immun. 1 (2009) 194–201.
[11] D. Bershadsky, U. Glück, O.N. Denisenko, T.V. Sklyarova, I. Spector, A. Ben-Ze'ev, The state of actin assembly regulates actin and vinculin expression by a feedback loop, J. Cell Sci. 108 (1995) 1183–1193.
[12] W. Moller, W.G. Krehling, M. Kohlhaufl, K. Haussinger, J. Heyder, Macrophage functions measured by magnetic microparticles in vivo and in vitro, J. Magn. Magn. Mater. 225 (2001) 218–225.
[13] R.D. Eicher, M.A. Salami, P.R. Wood, M.A. Mullbacher, The effect of gliotoxin on macrophage function, Int. J. Immunopharm. 8 (1986) 789–797.
[14] P. Mowlds, C. Coates, J. Renwick, K. Kavanagh, Dose-dependent cellular and humoral responses in *Galleria mellonella* larvae following glucan inoculation, Microb. Infect. 12 (2010) 146–153.
[15] T. Mitchell, A. Lo, M. Logan, P. Lacy, G. Eitzen, Primary granule exocytosis in human neutrophils is regulated by Rac-dependent actin remodeling, Am. J. Physiol. 295 (2008) 1354–1365.
[16] C.A. Wang, S.Y. Du, C.W. Lin, H.W. Fu, Helicobacter pylori neutrophil-activating protein promotes myeloperoxidase release from human neutrophils, Biochem. Biophys. Res. Commun. 377 (2008) 52–56.
[17] A. Zdybicku-Barabas, M. Cytrynska, Involvement of apolipophorin III in antibacterial defense of *Galleria mellonella* larvae, J. Biochem. Mol. Biol. 158 (2011) 90–98.
[18] H. Kim, H.J. Je, S.Y. Park, I.J. Lee, B.J. Jin, H.K. Yun, C.Y. Yun, Y.S. Han, Y.S. Kang, S.J. Seo, Immune activation of apolipophorin-III and its distribution in hemocyte from Hyphantria cunea, Insect Biochem. Mol. Biol. 34 (2004) 1011–1023.
[19] M. Locke, C. Ketola-Pirie, H. Leung, H. Nichol, Vacuolar apoferritin synthesis by the fat body of an insect (*Calpodes ethlius*), J. Insect Physiol. 34 (1991) 297–309.
[20] A. Standish, J. Weisner, Human neutrophils kill *Streptococcus pneumoniae* via serine proteases, J. Immunol. 185 (2010) 2602–2609.
[21] P. Paily, B. Agiesh Kumar, K. Balaraman, Transferrin in the mosquito, *Culex quinquefasciatus* Say (Diptera: Culicidae), up-regulated upon infection and development of the filarial parasite, *Wuchereria bancrofti* (Cobbold) (Spirurida: Onchocercidae), Parasitol. Res. 101 (2007) 325–330.
[22] C. Comera, K. Andre, K. Laffitte, X. Collet, P. Galtier, I. Maridonneau-Parini, Gliotoxin from *Aspergillus fumigatus* affects phagocytosis and organization of the actin cytoskeleton by distinct signaling pathways in human neutrophils, Microb. Infect. 9 (2007) 47–54.
[23] H. Hamamoto, K. Kurohawawa, C. Kaito, K. Kamura, I.M. Razanajatovo, et al., Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms, Antimicrob. Agent Chemother. 48 (2004) 774–779.
[24] K. Kavanagh, J. Fallon, *Galleria mellonella* larvae as models for studying fungal virulence, Fungal Biol. Revs. 24 (2010) 79–83.
[25] S. Tsunawaki, L. Yoshida, S. Nishida, T. Kobayashi, T. Shimoyama, Fungal metabolite gliotoxin inhibits assembly of the human respiratory burst NADPH oxidase, Infect. Immun. 72 (2004) 3373–3382.
[26] E. Mylonakis, R. Moreno, J.B. Khoury, A. Indrum, J. Heikman, et al., *Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis, Infect. Immun. 73 (2005) 3842–3850.