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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.
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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

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