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Characterisation of immune responses in the pea aphid, *Acyrthosiphon pisum*

Alice M. Laughton [a,*], Justine R. Garcia [a], Boran Altincicek [a,c], Michael R. Strand [b], Nicole M. Gerardo [a]

[a] Biology Department, Emory University, O. Wayne Rollins Research Center, 1510 Clifton Road NE, Atlanta, GA, 30322, USA
[b] Department of Entomology, University of Georgia, Athens, GA, 30602, USA
[c] Rheinische Friedrich-Wilhelms-University of Bonn, INRES-Phytomedicine, Nussallee 9, D-53115 Bonn, Germany

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ABSTRACT

The innate immune system of insects provides effective defence against a range of parasites and pathogens. The pea aphid, *Acyrthosiphon pisum*, is a novel study system for investigating host–parasite interactions due to its complex associations with both well-characterised bacterial symbionts and a diversity of pathogens and parasites, including several important biological control agents. However, little is known about the cellular and humoral immune responses of aphids. Here we identify three morphologically distinct types of haemocytes in circulation that we name prohemocytes, granulocytes and oenocytoids. Granulocytes avidly phagocytose Gram negative *Escherechia coli* and Gram positive *Micrococcus luteus* while oenocytoids exhibit melanotic activity. Prohaemocytes increase in abundance immediately following an immune challenge, irrespective of the source of stimulus. Pea aphids form melanotic capsules around Sephadex beads but do not form cellular capsules. We also did not detect any antimicrobial peptide activity in the haemolymph using zone of inhibition assays. We discuss these results in relation to recent findings from the pea aphid genome annotation project that suggest that aphids have a reduced immune gene repertoire compared to other insects.

1. Introduction

The innate immune system of insects consists of cellular and humoral components that provide defence against a diverse range of parasites including bacteria, fungi and parasitoids (e.g. Ashida and Brey, 1995; Muta and Iwanaga, 1996; Gillespie et al., 1997; Söderhäll and Cerenius, 1998; Schmid-Hempel, 2005). Recognition of a foreign entity like bacteria induces several immediate effector responses including clotting, encapsulation or nodule formation, phagocytosis, and melanisation (Gillespie et al., 1997; Söderhäll and Cerenius, 1998; Schmid-Hempel, 2005). These responses often clear a large proportion of bacteria within the first thirty minutes of infection (Haine et al., 2008). Subsequent inducible responses such as the production of antimicrobial peptides (AMPs) and lysozymes take longer to manufacture and are more costly, but are longer lasting in their effects (Boman and Hultmark, 1987; Eleftherianos et al., 2006; Lemaitre and Hoffmann, 2007; Haine et al., 2008; Ye et al., 2009). To date, little is known about how or whether immune responses are modified in insects with obligate and/or frequent associations with beneficial microbial symbionts.

Symbiotic relationships are ubiquitous in nature (Ruby et al., 2004). Aphids, like many animals, are dependent on beneficial microbes but must frequently deal with the consequences of harmful ones. The obligate intracellular Gram negative bacterium *Buchnera aphidicola* provides essential amino acids necessary for aphids, and, in turn, is compartmentalised within specialised bacteriocytes in the aphid haemocoel that afford it a protected environment in which to live (Griffiths and Beck, 1973; Douglas, 1998; Moran et al., 2005a). *Buchnera* is transmitted maternally to developing embryos or eggs, and as such is reliant on the aphid host for its survival (Douglas, 1998). Aphids are subject to attack from a range of parasites, including fungi and parasitoid wasps (Hufbauer, 2002; Snyder and Ives, 2003). Facultative secondary bacterial symbionts, which are found both in bacteriocytes and in the aphid haemocoel, can provide defence against some of these agents (Oliver et al., 2003; Scarborough et al., 2005). In addition, aphids vector a number of agriculturally detrimental plant viruses (Cuperus and Radcliffe, 1982; Ng and Perry, 2004). However, despite this complex relationship with beneficial and harmful microbes, work evaluating the cellular and humoral immune responses of aphids is currently limited (Henter and Via, 1995; Oliver et al., 2005; Altincicek et al., 2008; Gerardo et al., 2010). In this investigation we present results describing the basal immune responses of a clonal line of the pea aphid, *Acyrthosiphon pisum*, which carries the obligate *Buchnera* symbiont but does not harbour any facultative secondary symbionts (Moran et al., 2005b) or other microbes, allowing us to gain a measure of the immune responses of the aphid in its simplest symbiotic state.

* Corresponding author. Tel.: +1 404 727 9344; fax: +1 404 727 2880.
E-mail address: alice.laughton@emory.edu (A.M. Laughton).

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2. Materials and methods

2.1. Aphids and bacterial cultures

All assays were carried out on clonally produced female offspring from parthenogenetic females of the 5A0 line of pea aphids, which has been maintained as a laboratory stock line since collection in 1999 from Madison, WI. The line contains only the obligate symbiont, *Buchnera*, and no other known symbionts (Moran et al., 2005b). In addition, the results of some assays were verified in other aphid lines, as indicated below. All assays were carried out on adult aphids that had completed their final moult and begun clonally reproducing two days prior to experimentation. Aphids were kept in cages in a walk-in growth chamber and maintained on *Vicia faba* (fava bean) at 20 °C and 16L:8D. All experiments required that aphids be destructively sampled during data collection. Therefore, in experiments following immune responses over a time-course, a different cohort of aphids was sampled at each time point rather than collecting multiple samples from the same individual.

The Gram negative (genus *Enterobacter*) and Gram positive (genus *Bacillus*) bacteria used in the cell count, phenoloxidase and antimicrobial assays described below were originally isolated from pale and presumably sick laboratory pea aphids. Both bacterial strains, when alive, cause aphid mortality within several days after being fed or stabbed at low doses (Gerardo et al., 2010; unpublished data). Bacteria were stored as glycerol stocks and grown on Luria broth (LB) agar plates prior to use. A single colony selected from the plates was then cultured in LB broth (incubated overnight, 37 °C with shaking), adjusted to the correct colony density, and heat-killed before being used in the assays.

2.2. Haemocyte characterisation

The number and type of haemocytes found in pea aphids was unknown prior to this study. Although nomenclature differs somewhat between taxa, four main types of haemocytes are commonly recognised in insects on the basis of morphological, histochemical/antigenic and functional characteristics (Lackie et al., 1988; Tojo et al., 2000; Lavine and Strand, 2002; Ribeiro and Brehélin, 2006; Strand, 2008). Granulocytes are strongly adhesive cells whose primary function is phagocytosis, while plasmatocytes are adhesive and form cellular capsules. Oenocytoids contain phenoloxidase precursors involved in the production of melanin, and prohaemocytes are thought to be stem cells (Lackie et al., 1988; Lavine and Strand, 2002). In contrast, a study by Bensadia et al. (2006) with pea aphids reported only large globular "spherulocytes" adhered to parasitoid eggs, while Behera et al. (1999) reported four haemocyte types (prohaemocytes, granulocytes, plasmatocytes and adipohaemocytes) on the basis of morphology in the chrysanthemum aphid, *Macrosiphoniela sanborni*.

To characterise haemocyte types in the pea aphid, neat haemolymph was collected from a leg wound of individual aphids, via a glass capillary (0.141 mm × 32 mm, 2 μl vol.), and added to Grace's insect medium (20 μl, Sigma G8142) on a teflon ringed slide for analysis. Cells were observed in culture (room temp., 19 °C ± 2 °C) at 0, 10, 30 and 60 min after collection using DIC microscopy. Additional haemocyte samples were smeared onto glass slides and stained using a Diff-Quick stain kit (IMEB Inc.). Because some cell types were fragile and lysed using the collection methods outlined above, haemolymph samples were also collected from a leg bleed directly into a drop protruding from a pipette tip containing 12 μl of a methylene blue solution (2% diluted 1:10 in phosphate buffered saline (PBS, 150 mM NaCl, 10 mM Na_2HPO_4, pH 6.5)). The solution was then pipetted onto a slide and viewed immediately. Relative proportions of each haemocyte type were estimated and averaged over five samples. To ensure that any haemocytes identified were not specific to the single 5A0 aphid stock line tested or to adults only, haemocytes from adults of five other pea aphid stock lines (G6, LSR01, 7A, A2A and G15) and from juvenile 5A0 aphids were also examined. As with the 5A0 line, all of the additional lines contained only the obligate symbiont *Buchnera*, and no other known symbionts.

2.3. Cell counts

Cell counts were carried out over a 24-h (samples collected at the time points 0.5, 1, 5, 12, and 24 h post-challenge) and a 6-day (samples collected at the same time of day, each day up to 6 days post-challenge) time course. Aphids were split into four treatment groups: a full control (*No Challenge*), wounding control (stab with a sterile 0.15 mm diameter metal needle, *Stab*), and two immune challenged groups receiving a stab with a metal needle dipped in a heat-killed solution of either Gram negative (*Gram −ve*) or Gram positive (*Gram +ve*) bacterial pathogens at a concentration of 7×10^6 cfu ml^{-1}. Aphids in all treatment groups (including *No Challenge*) were handled similarly, with each being held in a Petri dish for approximately 45 min while treatments were administered and a brief recovery period given. Post challenge, aphids were returned to fresh plants (7 aphids per plant) and incubated (20 °C and 16L:8D) before haemolymph was collected. Each sample of pooled neat haemolymph (0.25 μl) was collected from leg wounds of two aphids using a glass capillary. Samples were immediately smeared onto a slide, fixed and stained with a Diff-Quick stain kit, and then the number of each haemocyte type was recorded ($n = 4$–7 per time:treatment group combination). Due to the time taken to process samples, the experiment was split with identical replicates conducted on two separate days. The date was included as a co-factor in the analysis to control for any variation due to collection day.

2.4. Phagocytosis

Phagocytosis is a multistep endocytic process by which haemocytes recognise, bind and ingest small foreign entities like bacteria (Gillespie et al., 1997). Phagocytic activity was assayed *in vivo* using heat killed *Escherichia coli* (Gram negative) or *Micrococcus luteus* (Gram positive) labeled with fluorescein isothiocyanate (FITC) (Beck and Strand, 2005). We injected 1×10^3 of each bacterium per aphid in a volume of 0.25 μl of PBS using a glass needle mounted on a micromanipulator. Ten aphids were tested with each bacterium. Aphids were returned to fresh plants for one hour followed by bleeding directly into 20 μl Excell 420 medium (Thermo) onto a teflon ringed slide. Haemocytes were allowed to settle for 15 min, and then fixed by adding 20 μl of 10% formalin in PBS. The combined volume of medium and fixative was removed after 1 min, and replaced with 20 μl 10% formalin in PBS. After 10 min, 20 μl of PBS containing 0.1% Triton X-100 (phosphate buffered Triton, PBT) was added, and the sample allowed to sit for another 5 min. Finally, most of the liquid was removed and replaced with 20 μl PBT plus 2 μg ml^{-1} DAPI (4′,6-diamidino-2-phenylindole in dH_2O). A cover slip was added and samples viewed under epifluorescent and phase contrast optics. Samples were scored for the presence or absence of phagocytised bacteria, and the identity of the haemocyte type that was phagocytic.

2.5. Encapsulation

Cellular encapsulation is a defence response against large foreign targets such as nematodes, cestodes, and parasitoid eggs that cannot be phagocytised by individual haemocytes (Dunn, 1986; Lackie et al., 1988). During a cellular encapsulation response, multiple haemocytes bind to the foreign object to form an overlapping sheath of cells which sometimes also melanise (Pech