Cellular Microbiology (2007) **9**(5), 1137–1147
doi:10.1111/j.1462-5822.2006.00855.x
First published online 12 December 2006

The immunoglobulin family protein Hemolin mediates cellular immune responses to bacteria in the insect *Manduca sexta*

Ioannis Eleftherianos,[1] Fırat Gökçen,[1,2] Gabriella Felföldi,[1,3] Peter J. Millichap,[1] Tina E. Trenczek,[2] Richard H. ffrench-Constant[1] and Stuart E. Reynolds[1]*

[1]*Department of Biology and Biochemistry, University of Bath, Claverton Down, UK.*
[2]*Institut für Allgemeine und Spezielle Zoologie, Justus-Liebig-Universität, Giessen, Germany.*
[3]*Department of Biochemistry, Eötvos Lörand University, Budapest, Hungary.*

Summary

Bacterial recognition in the lepidopteran insect, *Manduca sexta*, is mediated by pattern recognition proteins including Hemolin, Peptidoglycan recognition protein (PGRP) and Immulectin-2. These proteins bind to molecular patterns present on the surface of bacteria and trigger a protective response involving humoral and cellular reactions. Cellular mechanisms mediated by haemocytes include phagocytosis, encapsulation, and the formation of melanotic nodules. Here, we show that a non-pathogenic strain of *Escherichia coli* induces mRNA transcription and protein expression of Hemolin and PGRP but not Immulectin-2 in *Manduca* haemocytes. This upregulation can be effectively prevented (knocked-down) using RNA interference (RNAi) following injection of double-stranded (ds) RNA. Knock-down of Hemolin significantly decreased the ability of insects to clear *E. coli* from the haemolymph and caused a reduction in the number of free haemocytes. RNAi of Hemolin reduced the ability of haemocytes to engulf bacteria through phagocytosis and to form melanotic nodules *in vivo*. Importantly, washed haemocytes taken from RNAi-treated insects showed reduced ability to form microaggregates around bacteria *in vitro*. This shows that the immune function affected by RNAi knock-down of Hemolin is intrinsic to the haemocytes. In contrast, RNAi of PGRP had no effect on any of these cellular immune functions. These results demonstrate the vital role of Hemolin in *Manduca* cellular immune responses.

Received 4 August, 2006; revised 7 October, 2006; accepted 9 October, 2006. *For correspondence. E-mail S.E.Reynolds@bath.ac.uk; Tel. (+44) 1225 386936; Fax (+44) 1225 386779.

© 2006 The Authors
Journal compilation © 2006 Blackwell Publishing Ltd

Introduction

The insect immune system responds to infection by mounting both humoral and cellular responses. Humoral defences include the production of antimicrobial peptides (Meister *et al*., 2000; Lowenberger, 2001), reactive oxygen or nitrogen species (Bogdan *et al*., 2000; Vass and Nappi, 2001), and the activation of complex enzymatic cascades that regulate coagulation and melanization of haemolymph (Muta and Iwanaga, 1996; Gillespie *et al*., 1997; Cerenius and Soderhall, 2004). Cellular defences, on the other hand, include haemocyte-mediated immune responses like phagocytosis, nodulation and encapsulation (Strand and Pech, 1995; Schmidt *et al*., 2001). Lepidopteran insects (butterflies and moths) have received considerable attention as tractable experimental models of infection and immunity processes in insects (Silva *et al*., 2002), especially cellular immune defences (Ratcliffe, 1993; Gillespie *et al*., 1997; Lavine and Strand, 2002; Au *et al*., 2004; Ribeiro and Brehélin, 2006).

Recognition of microbial infection is an essential first step in insect immunity (Leulier *et al*., 2003; Royet, 2004) and is mediated by pattern recognition proteins (PRP) that bind conserved pathogen-associated molecular pattern (PAMP) molecules produced by microorganisms like bacteria (Fearon, 1997). In mounting cellular responses to microbes, haemocytes may recognize non-self either directly, through interaction of PRP already located on haemocytes with molecules on surface of the invading organism, or indirectly, by recognition of PRP–PAMP complexes that previously formed in the plasma; inter- and intracellular signalling events then co-ordinate effector responses, like phagocytosis or nodule formation (Lavine and Strand, 2002).

In the lepidopteran model insect *Manduca sexta* several PRP have been identified including Hemolin, Peptidoglycan recognition proteins (PGRP) and various Immulectins (Yu *et al*., 2002). Hemolin, a member of the immunoglobulin superfamily (Sun *et al*., 1990; Ladendorff

and Kanost, 1991), is a pattern recognition molecule exclusive to Lepidoptera. It binds to bacterial surface components such as lipopolysaccharide and lipoteichoic acid (Daffre and Faye, 1997). Hemolin lacks bactericidal activity, but has been shown to agglutinate bacteria (Yu and Kanost, 2002). It also binds to haemocytes *in vitro*, thereby inhibiting spontaneous and induced haemocyte aggregation (Ladendorff and Kanost, 1991; Lanz-Mendoza *et al*., 1996; Bettencourt *et al*., 1999). Taken together, the characteristics of Hemolin described above, together with its structural similarity to known cell adhesion molecules, suggest that Hemolin has a role in cellular recognition and/or in cell–cell interactions during an infection. However, its precise role in immunity has not so far been elucidated.

In contrast, the other two PRP investigated here are likely to be principally concerned with humoral defences. Immulectin-2 (IML-2), a C-type lectin, binds to serine proteases in insect plasma that activate prophenoloxidase (proPO) to active phenoloxidase or PO (Kanost *et al*., 2004). IML-2 has been shown to be important in protecting against Gram-negative bacteria and may function by localizing the PO response to the surface of invading bacteria (Kanost *et al*., 2004). Finally, PGRPs recognize peptidoglycan in the bacterial cell wall. PGRP was first purified from silkworm haemolymph via its high-affinity binding to peptidoglycan and its ability to trigger peptidoglycan-dependent activation of the proPO cascade (Yoshida *et al*., 1996).

We have previously used RNA interference (RNAi) to confirm the functional role of all three of these pattern recognition proteins in the *Manduca* humoral immune response to injected bacteria. To do this we injected double-stranded (ds) RNA specific to the targeted gene into intact insect larvae and then challenged them with a pathogenic bacterium. RNAi knock-down of any one of Hemolin, PGRP or IML-2 resulted in increased susceptibility to the insect-specific pathogens *Photorhabdus luminescens* and *P. asymbiotica* (Eleftherianos *et al*., 2006a,b). Knock-down of IML-2 was shown to interfere with the humoral response by preventing normal activation of phenoloxidase (Eleftherianos *et al*., 2006a) but the roles of the other two PRP were not determined.

We previously showed that expression of all three PRP is strongly induced in *Manduca* fat body cells by injections of living Gram-negative bacteria, both pathogenic and non-pathogenic in their effects (Eleftherianos *et al*., 2006a,b). It has been previously reported that circulating haemocytes are also induced by immune challenge to produce Hemolin and PGRP but not Immulectin-2 (Wang *et al*., 1995; Yu and Kanost, 2000).

In this article, we confirm that Hemolin and PGRP, but not IML-2, are transcribed in haemocytes in response to the injection of live Gram-negative bacteria. We show that expression of these two PRP in haemocytes can be knocked-down using RNAi, and use this approach to reveal directly the functional role of Hemolin, but not PGRP, in mediating haemocyte aggregation, nodule formation and phagocytosis. It is evident that Hemolin plays a central role in lepidopteran cellular immune defences.

Results

Reverse transcription-PCR and RNAi of immune genes in Manduca *haemocytes*

In order to determine if the recognition protein-encoding genes of *Manduca* were upregulated post infection, we looked at induction of *Hemolin*, *IML-2* and *PGRP* transcripts using reverse transcription (RT)-PCR in RNA extracted from haemocytes of infected larvae. We found that both *Hemolin* and *PGRP* were transcribed in haemocytes in response to *Escherichia coli* 18 h post injection, but that *IML-2* mRNA was undetectable. Controls injected with phosphate-buffered saline (PBS), or left untreated, showed all three transcripts were absent (Fig. 1A). Hemolin and PGRP proteins were also detectable via Western analysis in extracts of haemocytes from *E. coli* injected insects, but not in controls (Fig. 1B).

To knock-down transcription of *Hemolin* and *PGRP* in haemocytes, we pre-treated animals with dsRNAs specific to these genes. In each case, 6 h after RNAi treatment, and 18 h after bacterial infection, the mRNA level of the relevant PRP, as detected by RT-PCR, was strongly reduced in haemocytes (Fig. 2A). To confirm that this decrease in *Hemolin* and *PGRP* mRNA led to a similar decrease in protein expression, we carried out Western blots (Fig. 2B), confirming that RNAi also markedly lowered the level in haemocytes of both Hemolin and PGRP. All non-infected and untreated controls showed undetectable levels of *Hemolin* or *PGRP* mRNA or protein. We also showed that the RNAi effect was gene specific. Not only was the knock-down of each PRP specific to the dsRNA reagent used, but a control dsRNA from an irrelevant gene (dsCON) had no effect on PRP mRNA or protein levels. Transcription of the ribosomal protein rpS3 (Jiang *et al*., 1996), used as a loading control, was unaffected by any of the dsRNA reagents. We also performed negative controls including injection of water instead of dsRNA, and injection of dsRNA, not followed by *E. coli* injection; in each of these cases there was no detectable gene transcription or expression of either *Hemolin* or *PGRP*.

In interpreting these results it must be borne in mind that all three of the genes investigated are also expressed in fat body. They are induced by the same stimuli, and secreted in to the haemolymph (Eleftherianos *et al*.,

© 2006 The Authors

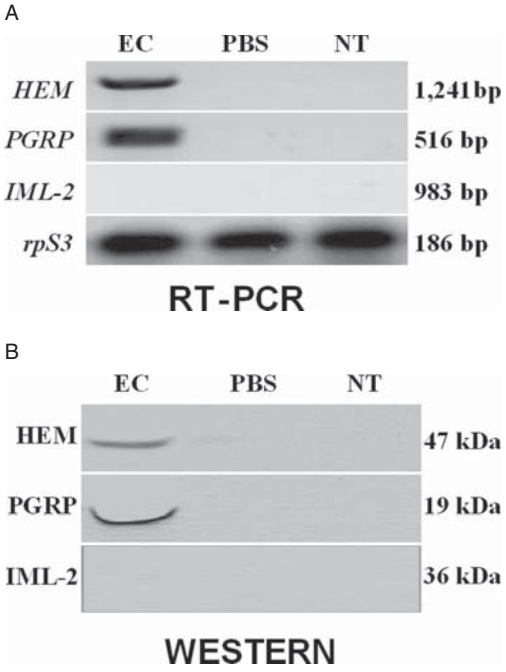

Fig. 1. A non-pathogenic strain of *E. coli* (DH5α) upregulates recognition genes in *Manduc*a haemocytes. (A) RT-PCR and (B) Western analysis of *Hemolin* (*HEM*), Immulectin (*IML-2*) and peptidoglycan recognition protein (*PGRP*) encoding genes from haemocytes extracted from insects infected with *E. coli* (EC). Note transcription and protein expression of *HEM* and *PGRP* following injection of 10^6 bacterial cells. Note the absence of *IML-2* gene transcription and expression as well as in animals injected only with phosphate-buffered saline (PBS) or left untreated (NT). A loading control (*Manduca* ribosomal protein S3 – *rpS3*) was used to ensure equal loading in each lane. Transcription and expression levels were assessed in duplicate for each treatment; results for single individuals are shown.

2006a,b). We cannot exclude the possibility that plasma proteins may be imported into or adhere to haemocytes. Thus, in each case some of the protein seen in the Western blots of haemocytes may have been derived from fat body rather than the haemocytes themselves. However, our previous results (Eleftherianos *et al.*, 2006a,b) clearly show that levels of Hemolin and PGRP protein in haemolymph plasma (regardless of where the proteins were synthesized) are strongly reduced by the RNAi treatment.

Effect of RNAi on the survival of E. coli *within infected* Manduca

In order to document how RNAi knock-down of immune genes in *Manduca* haemocytes affects bacterial survival, we injected a dsRNA produced from the coding sequences of either *Hemolin* or *PGRP* prior ($t = -6$ h) to injection ($t = 0$ h) with non-pathogenic bacteria (~10^6 *E. coli*); subsequently ($t = +18$ h), we recorded both the total number of haemocytes per insect and the number of recoverable bacteria from the haemolymph of the insect. Following injection of *E. coli*, the density of circulating haemocytes increased by 89% from a resting level of 8×10^5 cells ml^{-1} to an increased level of 1.6×10^6 cells ml^{-1}. This corresponds to approximately 4×10^5 and 8×10^5 haemocytes per insect respectively (Fig. 3A). Control treatments (injection of control dsRNA or PBS instead of the relevant PRP dsRNA reagent) had no effect on the total number of haemocytes. However, injection of dsHEM 6 h before the *E. coli* treatment significantly decreased (by about 30%) haemocyte numbers as measured at 18 h after the bacteria infection (one-way ANOVA, d.f. = 4,25, $F = 36.05$; $P < 0.001$; Tukey post hoc test, $P < 0.05$), and this effect was not observed when insects

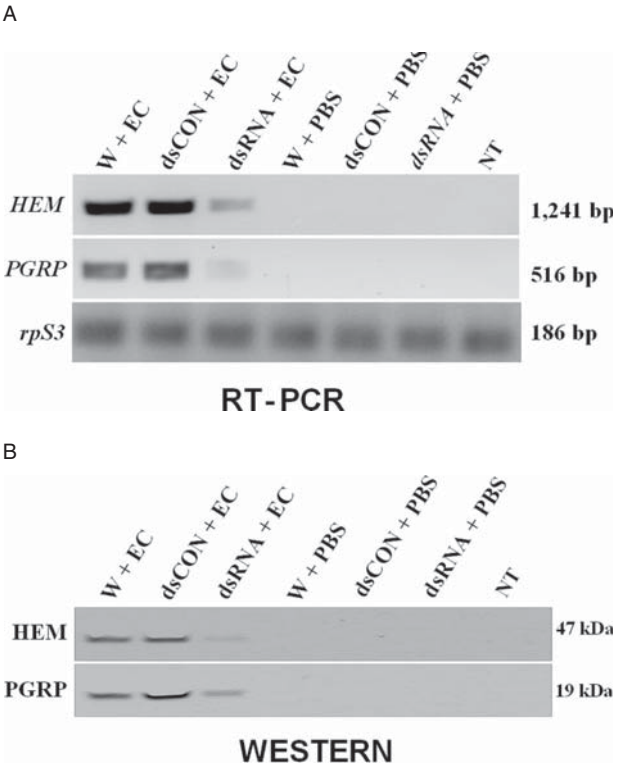

Fig. 2. RNAi-mediated knock-down of immune recognition transcripts and their encoded proteins.
A. RT-PCR shows that *E. coli*-induced transcription of both immune recognition genes (*HEM* and *PGRP*) can be knocked down by prior injection of the cognate dsRNA. In contrast, the control dsRNA dsCON has no effect on immune gene transcription. Note the absence of transcription of either gene in the controls.
B. Western analysis shows that RNAi of both recognition genes also reduces the level of the associated proteins found in the haemocytes. However, protein expression of either recognition gene is not reduced in insects pre-injected with dsCON. Again note the absence of recognition protein induction in the controls. The specificity of staining was checked by omitting the primary antibody, which led to complete absence of bands (result not shown).
RT-PCR and Western expression levels were assessed in duplicate for each treatment; results for single individuals are shown. W, water; EC, *E. coli*; PBS, saline; NT, no treatment.

© 2006 The Authors
Journal compilation © 2006 Blackwell Publishing Ltd,

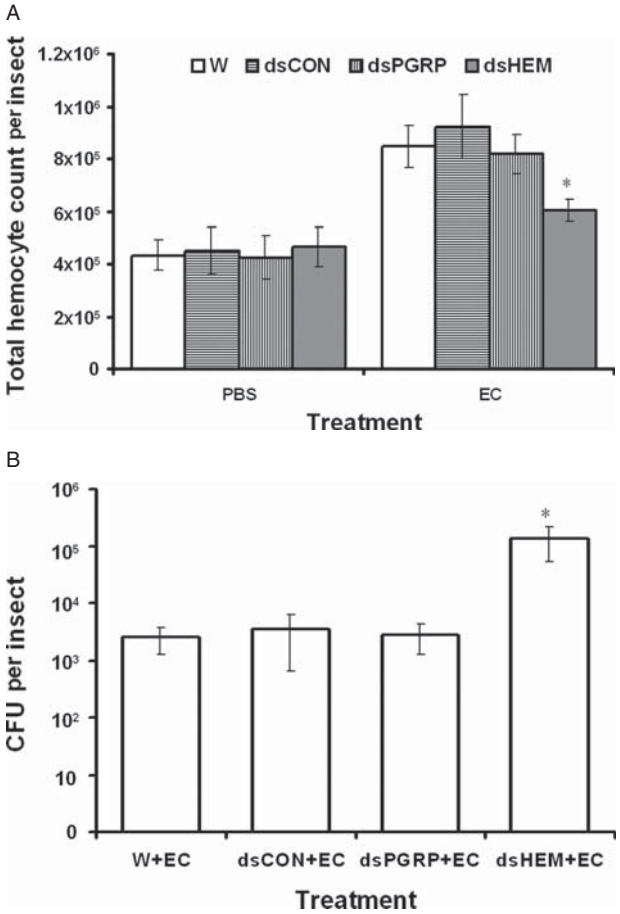

Fig. 3. Numbers of haemocytes and bacteria during *E. coli* infection of *Manduca*.
A. Estimated total free haemocyte count 18 h following injection of *E. coli*. Larvae were injected with 10^6 bacteria and then bled 18 h later to determine the number of free haemocytes. Note the increase in free haemocytes after *E.* coli injection and the decline in haemocytes following pre-injection with dsRNA of Hemolin. Bars show means ± SD ($n = 6$). The asterisk shows a value that is significantly different from the other treatments (ANOVA).
B. Numbers of recoverable bacteria (cfu, colony-forming units) 18 h following injection of *Manduca* larvae with *E. coli*. Larvae were injected with 10^6 bacterial cells and then bled 18 h later to determine the number of recoverable cfu in the insect haemolymph. Note that *E. coli* grow faster in insects in which Hemolin has been knocked down by RNAi. Bars show means ± SD ($n = 5$). The asterisk shows a value that is significantly different from the other treatments (ANOVA).
Abbreviations as in Fig. 2.

were injected with either dsCON or dsPGRP. At the same time, a significantly larger number of bacteria (an increase of around 500-fold) was recovered from insects injected with dsHEM prior to the *E. coli* infection compared with insects pre-injected with water, dsCON or dsPGRP (Fig. 3B) (one-way ANOVA, d.f. = 3,16, $F = 12.702$, $P < 0.001$; Tukey post hoc test, $P < 0.05$). No significant differences in recoverable bacteria were found between the various control treatments.

RNAi of Hemolin reduces nodulation, haemocyte aggregation and phagocytosis

To investigate the effect of the RNAi treatments on key cellular immune functions, we treated *Manduca* caterpillars with dsRNA specific to *Hemolin* or *PGRP*. First, we tested whether RNAi knock-down of Hemolin or PGRP affected the ability of *Manduca* haemocytes to phagocytose *E. coli* cells *in vivo*. We counted ingested labelled bacteria within haemocytes from either dsRNA or water pre-treated insects (Fig. 4A). Most phagocytosed bacteria were within only a small number of cells, which, like the hyperphagocytes identified by Dean *et al*. (2004), contained very large numbers of engulfed bacteria. We found that injection of dsHEM before injecting *E. coli* significantly reduced the number of bacteria within these phagocytic cells (one-way ANOVA, d.f. = 3,16, $F = 10.379$, $P < 0.001$; Tukey post hoc test, $P < 0.05$), but that other treatments, including RNAi knock-down of PGRP, did not. Confocal microscopy of TRITC phalloidin-stained haemocytes containing engulfed labelled bacteria allowed us to confirm that these bacteria were indeed located within the haemocytes (Fig. 4B).

Next, we looked at the effects of RNAi on nodule formation *in vivo* following injection of *E. coli* (Fig. 5A and B). There was a significant decrease in the number of melanotic nodules formed in insects pre-treated with dsHEM compared with either PGRP dsRNA or the various controls (one-way ANOVA, d.f. = 3,16, $F = 10.309$, $P = 0.001$; Tukey post hoc test, $P < 0.05$).

Finally, we examined the ability of washed haemocytes to form aggregates around *E. coli* cells when exposed to them *in vitro* (Fig. 6A and B). Similarly, RNAi knock-down of Hemolin was the only treatment to show a significant reduction in microaggregates (one-way ANOVA, d.f. = 4,20, $F = 18.179$, $P < 0.001$; Tukey post hoc test, $P < 0.05$).

Discussion

Recognition of microorganisms by pattern-recognition receptors is a crucial function of the innate immune system of vertebrates and invertebrates (Janeway, 1992). Pattern-recognition receptors identified in *Manduca* include Hemolin, C-type lectins, β-1,3-glucan-binding proteins and peptidoglycan-binding proteins (Yu *et al*., 2002; Kanost *et al*., 2004). Hemolin is the major protein synthesized in fat body in response to both Gram-negative and Gram-positive bacteria in lepidopteran insects (Andersson and Steiner, 1987; Ladendorff and Kanost, 1990), suggesting that it has an important function in the immune response of these insects. Hemolin has been shown to bind to *E. coli* (Sun *et al*., 1990; Zhao and Kanost, 1996) and to increase the association of *E. coli* with haemocytes (Kanost and Zhao, 1996). Similarly, PGRP is rapidly induced to high

© 2006 The Authors
Journal compilation © 2006 Blackwell Publishing Ltd,

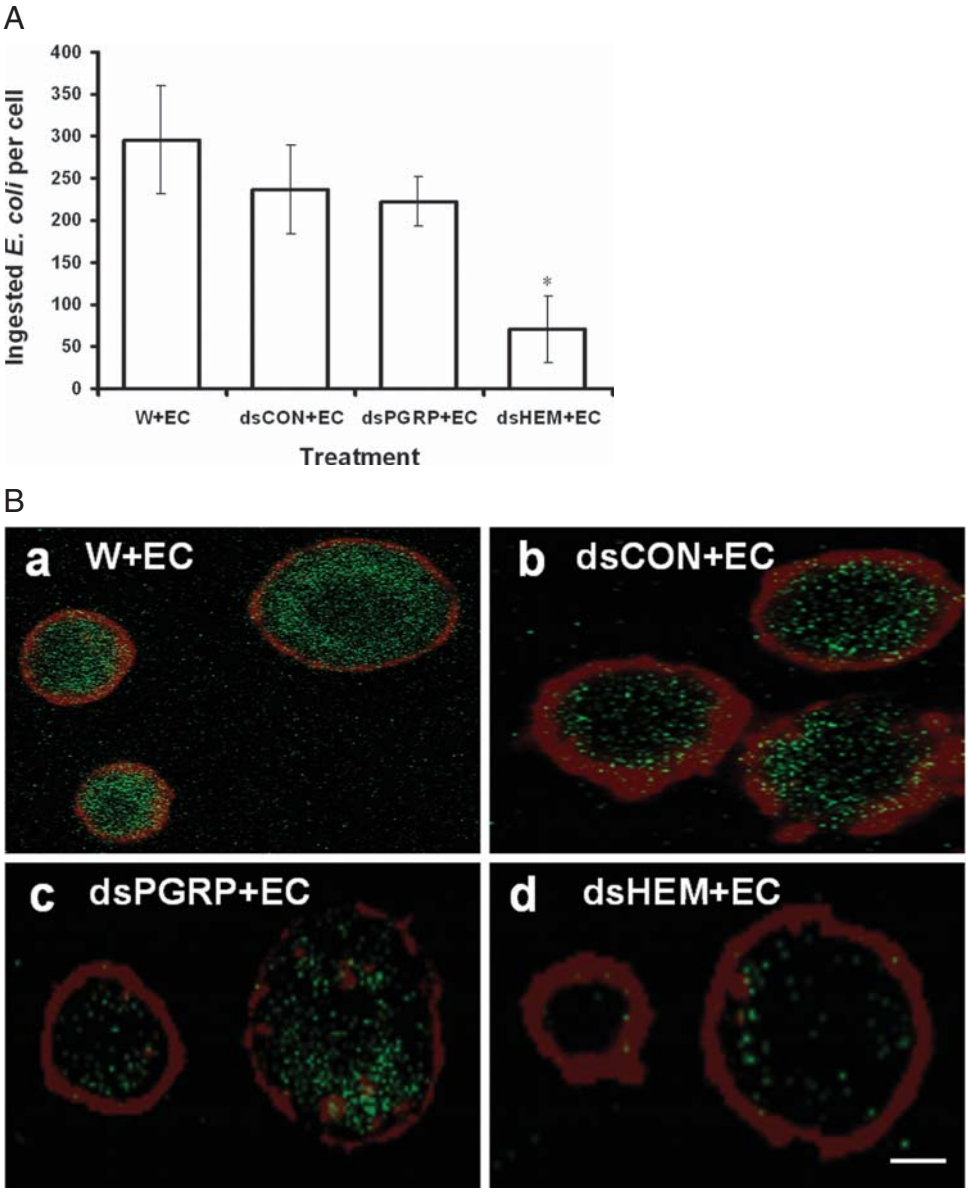

Fig. 4. Effect of RNAi-mediated *Hemolin* knock-down on the ability of *Manduca* haemocytes to phagocytose *E. coli*.
A. Histogram showing numbers of *E. coli* cells phagocytosed by hyperphagocytic cells during 18 h of bacterial infection, which was initiated 6 h after DMPC water or dsRNA injection. Bars show means ± SD ($n = 5$ insects, in each of which 20 haemocytes were counted). The asterisk shows a value that is significantly different from the other treatments (ANOVA).
B. *E. coli* bacteria visualized in *Manduca* haemocytes. Green shows bacteria cells; red shows cell surface staining with the monoclonal antibody MS77, used here as a counterstain. Note the decrease in the number of bacteria contained in haemocytes from insects injected with dsHEM prior to the *E. coli* infection, compared with the other treatments. Scale bar, 5 μm.
Abbreviations as for Fig. 2.

concentration levels in haemolymph following bacterial challenge (Yu *et al*., 2002). However, there has been no detailed investigation of the participation and role of these recognition proteins in haemocytic reactions responsible for bacterial clearance *in vivo*.

Fat body is a major site of Hemolin, PGRP and Immulectin-2 expression (Kanost *et al*., 2004) in *Manduca*. Our results confirmed that circulating haemocytes are induced by bacteria to produce Hemolin and PGRP but not Immulectin-2 (Wang *et al*., 1995; Yu and Kanost, 2000). We went on to investigate the roles of Hemolin and PGRP in *Manduca* immunity, with particular emphasis on cellular defences, by interfering with the induced expression of these proteins.

We have recently used RNAi (Carthew, 2001) to probe the roles of a number of immune-related genes in

© 2006 The Authors
Journal compilation © 2006 Blackwell Publishing Ltd,

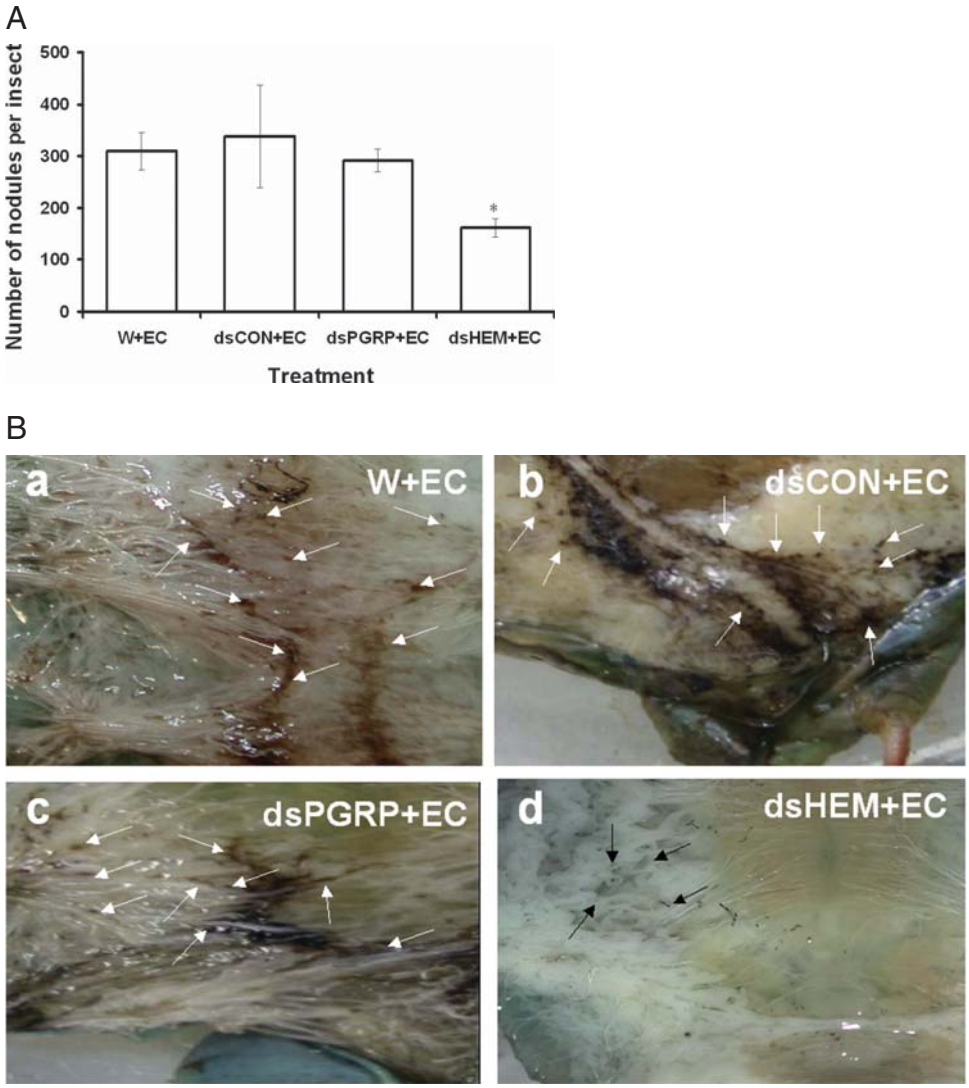

Fig. 5. A. Number of melanotic nodules in *Manduca* infected with *E. coli*. Insects were injected with DMPC-treated water (W) or dsRNAs 6 h before injected with 10^6 *E. coli* cells (EC), and left for 18 h before being dissected to determine the number of nodules located in the internal tissues. Bars show means ± SD ($n = 5$). The asterisk shows a value that is significantly different from the other treatments (ANOVA).
B. Formation of melanotic nodules in tissues of dissected *Manduca* larvae for each treatment. Note the reduction in melanotic nodules in caterpillars pre-injected with dsHEM compared with the water-, dsCON- or dsPGRP-pre-treated insects.

Manduca. We found that recognition protein and effector genes are strongly expressed in fat body following exposure to the insect pathogen *Photorhabdus*, and that RNAi knock-down results in faster death when previously uninfected or *E. coli* pre-infected insects are challenged with this pathogen (Eleftherianos *et al*., 2006a,b).

In the present study we have shown that dsRNA injection is also effective in silencing the expression of immune-related genes in *Manduca* haemocytes. RNAi treatment strongly repressed the expression of both Hemolin and PGRP, as shown by reductions in levels of both mRNA and protein. Several different controls (e.g. a control dsRNA; injecting specific dsRNAs without subsequent infection, or no treatment) were all consistent with specific suppression of the targeted gene. Importantly, we did not observe any case in which administration of a dsRNA reagent alone elicited expression of the targeted gene. This contrasts with the study of Hirai *et al*. (2004), in which upregulation of Hemolin expression in *Hyalophora cecropia* pupae was reported in response to dsHEM or the control dsGFP reagent. The reasons for the effect observed by Hirai *et al*. (2004) remain unknown, but the species and stage of insect were different.

RNAi knock-down of Hemolin caused significant differences in haemocyte responses to *E. coli*, but knock-down of PGRP did not have this effect. Clearance of bacterial cells from the haemolymph of dsHEM injected insects was significantly slower than controls, and the number

© 2006 The Authors

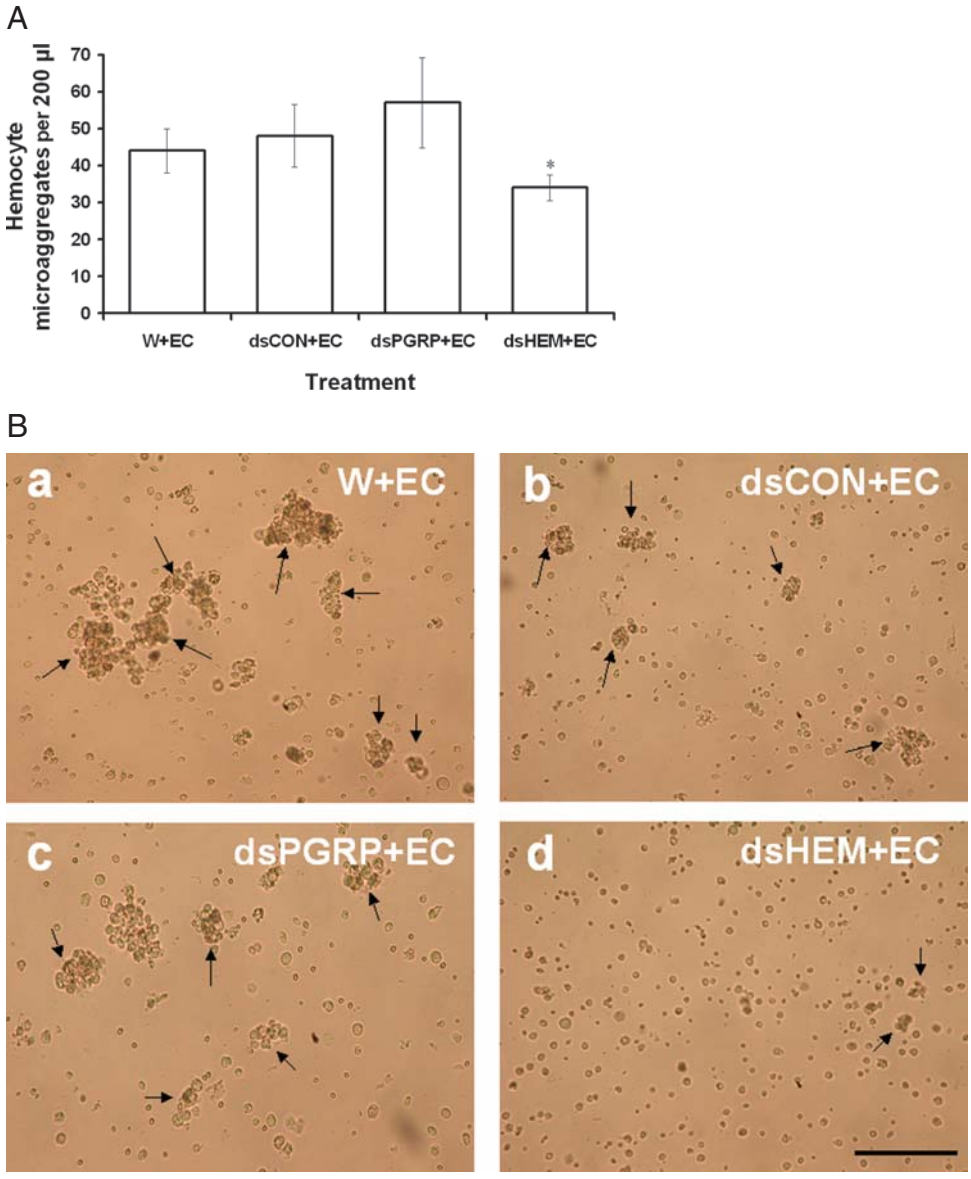

Fig. 6. RNAi of *Hemolin* decreases haemocyte aggregation *in vitro*.
A. Number of haemocyte microaggregates in haemolymph taken from uninfected *Manduca*, to which *E. coli* (EC) were added *in vitro*. Day 0 fifth-instar caterpillars were injected with DMPC water (W) or dsRNAs; 18 h later they were bled and haemocytes were collected. A suspension of 10^7 *E. coli* cells ml^{-1} was added to 10^6 haemocytes in a 1:2 ratio and following incubation for 15 min, the number of microaggregates was counted using an inverted microscope. Bars show means ± SD ($n = 5$). The asterisk shows a value that is significantly different from the other treatments (ANOVA).
B. Appearance of haemocyte microaggregates formed *in vitro* for each treatment. Note the reduction in microaggregates, when caterpillars were injected with dsHEM prior to the bacterial infection, in comparison with the water-, dsCON- or dsPGRP-pre-treated insects. Scale bar, 20 µm.

of colony-forming units (cfu) recovered from the treated insects was increased by about 500×. The increase in the number of free haemocytes that occurs in *E. coli*-treated controls was decreased by about 30%, but was not completely abolished, in the Hemolin knock-down insects. Bacterial clearance and haemocyte counts in PGRP knock-down insects were not different from the various controls. The mechanism of Hemolin's effect on the change in haemocyte number is not clear. It is, however, significant that Hemolin can reduce haemocyte aggregation (Ladendorff and Kanost, 1991), which may have a bearing on the release of previously sequestered haemocytes from the tissues.

To investigate the effect of RNAi on phagocytic competence, we examined the ability of RNAi knock-down of Hemolin and PGRP to interfere with *in vivo* phagocytosis

© 2006 The Authors

of labelled *E. coli* bacteria. Our results showed that over the 18 h experimental period, RNAi knock-down of Hemolin in *Manduca* markedly reduced the phagocytosis of injected *E. coli* by *Manduca* haemocytes *in vivo*, whereas knock-down of PGRP had no measurable effect on phagocytosis of bacteria.

We also examined the role of Hemolin in nodule formation. Injection of *E. coli* cells induced the formation of a large number of melanotic nodules in the internal tissues of *Manduca*. The number of nodules formed was significantly suppressed by pre-treating the insects with dsHEM, but not by dsPGRP or dsCON.

The results discussed above provide evidence for the participation of Hemolin in both phagocytosis and melanotic nodule formation *in vivo*. It has previously been suggested (Yu and Kanost, 2002) that Hemolin may promote both of these processes through the known ability of the protein to bind to haemocytes *in vitro*, together with its ability to bind to and cause the aggregation of microorganisms *in vitro*. But does the Hemolin participating in these processes come from the haemocytes themselves or from the haemolymph plasma?

We addressed this question by examining the process of *E. coli*-induced aggregation *in vitro* using washed haemocytes. These cells had not been exposed to bacteria before bleeding. Haemocytes taken from dsHEM-treated insects and therefore lacking in Hemolin formed significantly fewer microaggregates when exposed to *E. coli in vitro*, and these contained fewer haemocytes than controls. This indicates that the Hemolin participating in microaggregate formation is present in or on the haemocytes themselves. Of course, this does not say whether the Hemolin in question was synthesized in haemocytes, in fat body or elsewhere. It has been previously shown that the addition of exogenous Hemolin had the opposite effect, inhibiting haemocyte aggregation *in vitro* (Ladendorff and Kanost, 1991; Lanz-Mendoza *et al*., 1996). The decrease in aggregation seen in our experiments may have been due to interference with bacterial recognition. In our protocol, haemocyte aggregation was provoked *in vitro* by the presence of *E. coli*. Exogenous Hemolin is known to associate with bacterial cell surfaces in a specific and saturable manner (Zhao and Kanost, 1996), so that in our experiments an RNAi-induced lack of haemocyte-associated Hemolin may have prevented normal aggregation because the bacteria were bound less well. This would be consistent with previous work if the reported inhibition of haemocyte aggregation by exogenous Hemolin were due to high-affinity competition for bacterial binding sites between Hemolin already present on the surfaces of haemocytes, and added Hemolin dissolved in plasma.

Alternatively, the RNAi-induced decrease in aggregation observed in our experiments may have been due directly to altered interactions between haemocytes. It is possible that haemocyte aggregation depends on homophilic adhesive interactions between Hemolin molecules on the surfaces of the cells (Zhao and Kanost, 1996). In this case, the inhibition of haemocyte aggregation observed in our experiments might have resulted from RNAi-induced lack of Hemolin on the cells, whereas the previously observed Hemolin-mediated inhibition of aggregation might have been caused by interactions between haemocyte surface Hemolin and exogenous Hemolin in plasma.

It is clear from the results presented here that the recognition protein Hemolin plays a key role in the cellular immune responses of *Manduca*, while PGRP appears to have little if any involvement with cellular immunity. Recognition proteins have been suggested to play individual but overlapping roles in activating the insect immune system (Yu *et al*., 2002). Our results are consistent with a model in which specific recognition proteins trigger specific aspects of the insect immune response, as illustrated by the role of Hemolin in key haemocyte functions. The role of PGRP in *Manduca* immunity is still not clear (Kanost *et al*., 2004), but our results suggest that this PRP is likely to be involved in humoral rather than cellular responses.

Despite our conclusion that Hemolin is largely concerned with cellular immune responses, it must be remembered that immune responses are complex and there are many opportunities for cellular and humoral responses to interact cooperatively to determine the insect's ability to resist infection by pathogens. Such interactions might arise at a level beyond phagocytosis and nodule formation, for example, by the release of cytokine-like immune signals into the plasma. Consistent with the presence of such interactions, we have previously noted that silencing any one PRP may have unexpectedly severe consequences for the ability to resist infection by *Photorhabdus* (Eleftherianos *et al*., 2006a).

Experimental procedures

Insects and bacteria

Larvae of the Tobacco Hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae) were maintained individually on a wheat germ-based artificial diet at 25°C and a photoperiod of 17 h light : 7 h dark (Reynolds *et al*., 1985). Newly moulted (day 0) fifth-stage larvae were used for all injection experiments. The DH5α strain of *E. coli* was used in all experiments. Details of injection techniques were as described by Eleftherianos *et al*. (2006a,b). In brief, dsRNA reagents (100 ng in 50 μl) were injected into day 0 fifth-instar *Manduca* larvae 6 h before chal-

© 2006 The Authors

lenge with bacteria. Haemolymph samples were taken 18 h after the immune challenge.

Reverse transcription-PCR, RNAi and Western blotting

Insects were bled (~500 μl haemolymph per insect) as described by Eleftherianos *et al*. (2006a,b). To isolate RNA, haemocytes (30 mg) were homogenized in TRI reagent (Sigma). RNA samples were further purified by the addition of RNase free DNase I (Invitrogen) 1 U μl^{-1} at 37°C for 1 h and the enzyme was subsequently inactivated by incubation for 10 min at 75°C. Lack of DNA contamination was confirmed in every RNA sample via 35 cycles of PCR (95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 2 min) using RNA as the template. Single-step reverse transcription (RT)-PCR was performed with the 'OneStep' RT-PCR kit (Qiagen). Each reaction was carried out in a 50 μl volume containing 0.6 μM of forward and reverse gene primers (see Eleftherianos *et al*., 2006a,b) and 2 μg of RNA template. All primers were specific with the exception of *PGRP*. In this case the primers used did not distinguish between two almost identical *PGRP* genes (*PGRP-1* and *PGRP-2*) present in the *Manduca* genome (Zhu *et al*., 2003). However, as the mature proteins encoded by these two genes are identical the results accurately reflect the presence of mRNAs encoding PGRP. Amplifications were performed on a PTC-100 thermal controller (MJ Research) under the following cycling conditions: RT at 50°C for 30 min, 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 45°C for 30 s and 72°C for 1 min with a final extension of 72°C for 10 min. RT-PCR control reactions for ribosomal protein S3 (rpS3) (Jiang *et al*., 1996) were performed as outlined above.

cDNAs were cloned and the cloned DNA was used as the template to produce dsRNA reagents specific to the desired genes as described in Eleftherianos *et al*. (2006a,b). As a negative dsRNA control we used a gene from a plant, *Manihot esculenta* catalase *CAT1* (GenBank Accession No. AF170272). The template for synthesis of dsCON was a pBluescript $IIKS^+$ plasmid containing the cloned gene, which was amplified by PCR using T3 (ATTAACCCTCACTAAAGGGA) and T7 (TAATACGACTCAC TATAGGG) sequencing primers, and dsCON was then synthesized as described above. For RNAi, 100 ng of the specific dsRNA (50 μl, 2 μg ml^{-1}) was injected into fifth-instar *Manduca* 6 h before challenge with *E. coli*. Controls involving dimethyl pyrocarbonate (DMPC) water without dsRNA in the primary injections and PBS without *E. coli* in the secondary injections were also used. After RNAi injection, treated insects were held at 25°C for 18 h and then bled in order to collect the haemocytes as before. RT-PCR reactions using extracted RNA as a template and suitable pair of primers were performed to determine mRNA transcription patterns of each gene.

For Western analysis, *Manduca* haemocyte samples were diluted in sample buffer (2× sample buffer: 0.1% Tris, 4% SDS, 5% 2-mercaptoethanol, 0.01% Coomassie blue G250, pH 6.8) and separated by SDS-PAGE using a 12% acrylamide separating gel and a 5% acrylamide stacking gel. After electrophoresis, haemocyte protein samples were stained with Coomassie blue or transferred electrophoretically (Mini Trans-Blot Transfer Cell, Bio-Rad) to PVDF membranes (Bio-Rad). The blots were then blocked in 5% skimmed milk powder (Marvel) in transblotting solution (TBS: 20 mM Tris, 0.9% NaCl, pH 7.2) for 12 h at 4°C. The blots were washed (three times for 10 min) in TBS followed by incubation in 3% Tween transblotting milk solution (TTBS: 20 mM Tris, 0.9% NaCl, 0.1% Tween-20, pH 7.2) containing 1/10 000 dilution of the appropriate primary antibody (anti-serum raised in rabbit reactive against *M. sexta* Hemolin, PGRP or Immulectin-2; all generous gifts from Professor Michael Kanost, Kansas State University, USA), for 1 h at room temperature. After subsequent washing (three times for 10 min in TTBS) the blots were incubated in 3% TTBS milk solution containing 1/10 000 dilution of horseradish peroxidase-labelled goat anti-rabbit IgG secondary antibody (Upstate) for 1 h at room temperature. After further washing the bound antibodies were detected using a chemiluminescence Western blotting kit (Visualizer, Upstate) and the membranes were exposed to X-ray film (Biomax, Kodak) for 1 s to detect the signals.

Counting haemocytes and bacteria

Day 0 fifth-instar *Manduca* were injected with DMPC-treated water or dsRNAs 6 h before the *E. coli* (10^6 cells) or PBS (0.15 M sodium chloride, 10 mM sodium phosphate buffer, pH 7.4) injection and 18 h later were bled in *Manduca*-buffered saline solution (MBS; 4 mM NaCl, 40 mM KCl, 18 mM $MgCl_2$·6H20, 1.5 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 192.8 mM sucrose, pH 4.5) and haemocytes were collected. Aliquots of haemolymph (50 μl) were immediately added to pre-chilled Grace's Insect Medium (GIM, Sigma) (450 μl). The total haemocyte count was determined microscopically at 100× using a Neubauer haemocytometer. The average of two counts per insect of three insects per treatment was calculated. Serial dilutions of the haemolymph were plated onto 2.5% LB agar plates and cfu recorded 24 h later, the average of five insects per treatment was calculated. The THC and cfu per caterpillar were calculated assuming 500 μl of haemolymph per insect.

Nodule formation

Day 0 fifth-instar *Manduca* larvae were injected with dsRNAs or DMPC water; after 6 h, a further injection of 10^6*E. coli* cells or PBS was made, as described above. Nodule formation was assessed after 18 h. Insects were immobilized on ice for 15 min before dissection under 1% (w/v) NaCl solution saturated with phenylthiourea (which prevented general post-dissection melanization). Melanized, dark nodules within the haemocoel were observed using a stereomicroscope and an approximate manual count of nodule number made with the help of a tally counter. In general it was easy to recognize individual nodules. Five insects were examined per treatment.

Haemocyte aggregation

Day 0 fifth-instar *Manduca* larvae were injected with dsRNAs or DMPC water and 18 h later were bled (~100 μl of haemolymph) into 1.5 ml polypropylene tubes containing MBS. Haemocytes were centrifuged at 200 g for 10 min at 4°C and the cell pellets were re-suspended in 200 μl of cold GIM. Cell density of the suspension was adjusted to 10^6 cells ml^{-1} using a haemocytometer. A suspension of *E. coli* bacteria in GIM was prepared containing approximately 10^7 cells ml^{-1}. One hundred microlitres of bacterial suspensions were mixed with 200 μl of haemocyte suspensions in 0.5 ml polypropylene tubes and

© 2006 The Authors
Journal compilation © 2006 Blackwell Publishing Ltd,

samples were incubated for 15 min on a shaker at slow speed (1 r.p.m.) and at room temperature. Following incubation, cell suspensions were transferred to individual wells of a 96-well microtitre plate and the microaggregates were counted using a Nikon TMS-F inverted microscope. Microaggregates of five or more cells were counted as incipient nodules. The mean of five insects per treatment was calculated.

Bacterial labelling

Escherichia coli were labelled using the 'BacLight' green bacterial stain kit (Molecular Probes). One millilitre of overnight bacterial culture (approximately 2×10^7 cells) was centrifuged at 10 000 *g* and 4°C in a microcentrifuge for 10 min. The supernatant was removed and the cells were resuspended in 1 ml of sterile PBS. This suspension was diluted to give approximately 10^6 cfu ml^{-1}, and 0.5 ml of this dilution was mixed with 1 μl of the BacLight 100 μM working dye solution [BacLight ampoules were previously mixed in 74 μl of dimethyl sulfoxide (DMSO) (Sigma) to prepare 1 mM stock solution]. The BacLight–cell mixture was incubated for 15 min in the dark at room temperature and kept at 4°C until use.

Haemocyte phagocytosis

The phagocytic competence of *Manduca* haemocytes was determined in reference to their ability to ingest *E. coli*. Labelled bacteria (~5×10^4 cells suspended in 50 μl of PBS) were injected as described above. Haemolymph was withdrawn after 18 h. Haemocyte monolayers were prepared as follows. RNAi-treated or DMPC water control-treated *Manduca* larvae were bled 18 h after the *E. coli* infection and approximately 200 μl of haemolymph was allowed to drip into 800 μl of ice-cold MBS solution in a microcentrifuge tube. The tube was immediately inverted to mix, and then centrifuged at 200 *g* for 5 min at 4°C. The supernatant was replaced with 200 μl of cold GIM and the haemocytes were gently resuspended using a cut pipette tip. Haemocyte density was adjusted to 10^6 cells ml^{-1} in GIM using a haemocytometer. Then, 10 mm coverslips were washed in 70% ethanol and placed centrally into each well of a 24-well plate. The resulting cell suspension (100 μl) was pipetted onto each coverslip and then left undisturbed for 30 min (at room temperature) to allow the haemocytes to settle and form a monolayer. The monolayers were washed gently with a few drops of GIM and removed to new wells containing 400 μl of fresh GIM. Monolayers were fixed in 2% (w/v) formaldehyde in PBS (made fresh from paraformaldehyde) for 15 min. Cells were washed twice in PBS and then blocked in 3% (w/v) bovine serum albumin (BSA) for 1.5 h at room temperature. The blocking solution was washed off and the monolayers were incubated overnight at 4°C in hybridoma supernatant containing the monoclonal antibody MS77, reported to be specific for *Manduca* plasmatocytes and spherule cells (Willott *et al*., 1994), without dilution. Cells were washed twice in PBS and then incubated for 20 min in the dark with secondary antibody solution (TRITC-conjugated goat anti-mouse IgG, Sigma) at 1:100 (v/v) dilution in 0.3% BSA/PBS. After four further washes in PBS, and once with distilled water, coverslips were mounted at room temperature in 5 μl of Mowiol-4-88 solution (Calbiochem) and stored at 4°C overnight. Control slides were incubated with the secondary antibody only. Phagocytosing haemocytes were examined using a Zeiss LSM-510 laser scanning confocal microscope.

Acknowledgements

We thank Sandra Barnes for rearing lots of *Manduca*. We also thank Michael Kanost (Kansas State University, Manhattan, Kansas) for generous gifts of antibodies against Hemolin and PGRP, and John Beeching and Simon Bull (University of Bath) for *CAT1*. This work was supported by a grant to S.E.R. and R.H. ff.-C. from the BBSRC Exploiting Genomics Initiative. F.G. and G.F. were Marie Curie Fellows of the European Commission.

References

Andersson, K., and Steiner, H. (1987) Structure and properties of protein P4, the major bacteria-inducible protein in pupae of *Hyalophora cecropia*. *Insect Biochem* **17:** 133–140.

Au, C., Dean, P., Reynolds, S.E., and ffrench-Constant, R.H. (2004) Effect of the insect pathogenic bacterium *Photorhabdus* on insect phagocytes. *Cell Microbiol* **6:** 89–95.

Bettencourt, R., Gunne, H., Gastinel, L., Steiner, H., and Faye, I. (1999) Implications of hemolin glycosylation Ca^{2+}-binding on homophilic and cellular interactions. *Eur J Biochem* **266:** 964–976.

Bogdan, C., Rollinghoff, M., and Diefenbach, A. (2000) Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr Opin Immunol* **12:** 64–76.

Carthew, R.W. (2001) Gene silencing by double stranded RNA. *Curr Opin Cell Biol* **13:** 244–248.

Cerenius, L., and Soderhall, K. (2004) The prophenoloxidase-activating system in invertebrates. *Immunol Rev* **198:** 116–126.

Daffre, S., and Faye, I. (1997) Lipopolysaccharide interaction with hemolin, an insect member of the Ig– superfamily. *FEBS Lett* **408:** 127–130.

Dean, P., Potter, U., Richards, E.H., Edwards, J.P., Charnley, A.K., and Reynolds, S.E. (2004) Hyperphagocytic haemocytes in *Manduca sexta*. *J Insect Physiol* **50:** 1027–1036.

Eleftherianos, I., Millichap, P.J., ffrench-Constant, R.H., and Reynolds, S.E. (2006a) RNAi suppression of recognition protein mediated immune responses in the tobacco hornworm *Manduca sexta* causes increased susceptibility to the insect pathogen *Photorhabdus*. *Dev Comp Immunol* **30:** 1099–1107.

Eleftherianos, I., Marokhazi, J., Millichap, P.J., Hodgkinson, A.J., Sriboonlert, A., ffrench-Constant, R.H., and Reynolds, S.E. (2006b) Prior infection of *Manduca sexta* with non-pathogenic *Escherichia coli* elicits immunity to pathogenic *Photorhabdus luminescens*: roles of immune-related proteins shown by RNA interference. *Insect Biochem Mol Biol* **36:** 517–525.

Fearon, D.T. (1997) Seeking wisdom in innate immunity. *Nature* **388:** 323–324.

Gillespie, J.P., Kanost, M.R., and Trenczek, T. (1997) Biological mediators of insect immunity. *Ann Rev Ent* **42:** 611–643.

© 2006 The Authors

Hirai, M., Terenius, O., Li, W., and Faye, I. (2004) Baculovirus and ds-RNA induce Hemolin, but not antibacterial activity, in *Antheraea pernyi*. *Insect Mol Biol* **13:** 399–405.

Janeway, C.A. (1992) The immune system evolved to discriminate infectious nonself from noninfectious self. *Immu Today* **13:** 11–16.

Jiang, H., Wang, Y., and Kanost, M.R. (1996) Primary structure of ribosomal proteins S3 and S7 from *Manduca sexta*. *Insect Mol Biol* **5:** 31–38.

Kanost, M.R., and Zhao, L. (1996) Insect hemolymph proteins from the immunoglobulin superfamily. *Adv Comp Env Physiol* **23:** 185–197.

Kanost, M.R., Jiang, H., and Yu, X.Q. (2004) Innate immune responses of a lepidopteran insect, *Manduca sexta*. *Immunol Rev* **198:** 97–105.

Ladendorff, N.E., and Kanost, M.R. (1990) Isolation and characterization of bacteria-induced protein P4 from hemolymph of *Manduca sexta*. *Arch Insect Biochem Physiol* **15:** 33–41.

Ladendorff, N.E., and Kanost, M.R. (1991) Bacteria induced protein P4 (hemolin) from *Manduca sexta*: a member of the immunoglobulin superfamily, which can inhibit hemocyte aggregation. *Arch Insect Biochem Physiol* **18:** 285–300.

Lanz-Mendoza, H., Bettencourt, R., Fabbri, M., and Faye, I. (1996) Regulation of the insect immune response: the effect of hemolin on cellular immune mechanisms. *Cell Immunol* **169:** 47–54.

Lavine, M.D., and Strand, M.R. (2002) Insect haemocytes and their role in immunity. *Insect Biochem Mol Biol* **32:** 1295–1309.

Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J.H., Caroff, M., Lee, W.J., *et al.* (2003) The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition. *Nat Immunol* **4:** 478–484.

Lowenberger, C. (2001) Innate immune response of *Aedes aegyptii*. *Insect Biochem Mol Biol* **31:** 219–229.

Meister, M., Hetru, C., and Hoffmann, J.A. (2000) The antimicrobial host defence of *Drosophila*. In *Origin and Evolution of the Vertebrate Immune System. Current Topics in Microbiology, 248.* Du Pasquire, L., and Litman, G.W. (eds). Berlin: Springer-Verlag, pp. 17–36.

Muta, T., and Iwanaga, S. (1996) The role of hemolymph coagulation in innate immunity. *Curr Opin Immunol* **8:** 41–47.

Ratcliffe, N.A. (1993) Cellular defense responses of insects: unresolved problems. In *Parasites and Pathogens of Insects. Parasites*, Vol. 1. Beckage, N.E., Thompson, S.N., and Federici, B.A. (eds). San Diego, CA: Academic Press, pp. 267–304.

Reynolds, S.E., Nottingham, S.F., and Stephens, A.E. (1985) Food and water economy and its relation to growth in 5th-instar larvae of the tobacco hornworm, *Manduca sexta*. *J Insect Physiol* **31:** 119–127.

Ribeiro, C., and Brehélin, M. (2006) Insect haemocytes: what type of cell is that? *J Insect Physiol* **52:** 417–429.

Royet, J. (2004) Infectious non-self recognition in invertebrates: lessons from *Drosophila* and other insect models. *Mol Immunol* **41:** 1063–1075.

Schmidt, O., Theopold, U., and Strand, M.R. (2001) Innate immunity and evasion by insect parasitoids. *Bioessays* **23:** 344–351.

Silva, C.P., Waterfield, N.R., Daborn, P.J., Dean, P., Chilver, T., Au, C.P.Y., *et al.* (2002) Bacterial infection of a model insect: *Photorhabdus luminescens* and *Manduca sexta*. *Cell Microbiol* **4:** 329–339.

Strand, M.R., and Pech, L.L. (1995) Immunological basis for compatibility in parasitoid–host relationships. *Ann Rev Entomol* **40:** 31–56.

Sun, S.C., Lindström, I., Boman, H.G., Faye, I., and Schmidt, O. (1990) Hemolin: an insect immune protein belonging to the immunoglobulin superfamily. *Science* **250:** 1729–1732.

Vass, E., and Nappi, A.J. (2001) Fruit fly immunity. *Bioessays* **51:** 529–535.

Wang, Y., Willott, E., and Kanost, M.R. (1995) Organization and expression of the hemolin gene, a member of the immunoglobulin superfamily in an insect, *Manduca sexta*. *Insect Mol Biol* **4:** 113–123.

Willott, E., Trenczek, T., Thrower, L.W., and Kanost, M.R. (1994) Immunochemical identification of insect hemocyte populations: monoclonal antibodies distinguish four major hemocyte types in *Manduca sexta*. *Eur J Cell Biol* **65:** 417–423.

Yoshida, H., Kinoshita, K., and Ashida, M. (1996) Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori*. *J Biol Chem* **271:** 13854–13860.

Yu, X.Q., and Kanost, M.R. (2000) Immulectin-2, a lipopolysaccharide-specific lectin from an insect, *Manduca sexta*, is induced in response to gram-negative bacteria. *J Biol Chem* **48:** 37373–37381.

Yu, X.Q., and Kanost, M.R. (2002) Binding of hemolin to bacterial lipopolysaccharide and lipoteichoic acid. An immunoglobulin superfamily member from insects as a pattern-recognition receptor. *Eur J Biochem* **269:** 1827–1834.

Yu, X.Q., Zhu, Y., Ma, C., Fabrick, J.A., and Kanost, M.R. (2002) Pattern recognition proteins in *Manduca sexta* plasma. *Insect Biochem Mol Biol* **32:** 1287–1293.

Zhao, L., and Kanost, M.R. (1996) In search of a function for hemolin, a hemolymph protein from the immunoglobulin super-family. *J Insect Physiol* **42:** 73–79.

Zhu, Y., Johnson, T.J., Myers, A.A., and Kanost, M.R. (2003) Identification by subtractive suppression hybridization of bacteria-induced genes expressed in *Manduca sexta* fat body. *Insect Biochem Mol Biol* **33:** 541–559.

© 2006 The Authors
Journal compilation © 2006 Blackwell Publishing Ltd,