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Journal of Insect Physiology 51 (2005) 505–512
Journal of Insect Physiology
www.elsevier.com/locate/jinsphys

Parasitization of *Manduca sexta* larvae by the parasitoid wasp *Cotesia congregata* induces an impaired host immune response

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Received 21 July 2004; accepted 9 November 2004

Abstract

During oviposition, the parasitoid wasp *Cotesia congregata* injects polydnavirus, venom, and parasitoid eggs into larvae of its lepidopteran host, the tobacco hornworm, *Manduca sexta*. Polydnaviruses (PDVs) suppress the immune system of the host and allow the juvenile parasitoids to develop without being encapsulated by host hemocytes mobilized by the immune system. Previous work identified a gene in the *Cotesia rubecula* PDV (CrV1) that is responsible for depolymerization of actin in hemocytes of the host *Pieris rapae* during a narrow temporal window from 4 to 8 h post-parasitization. Its expression appears temporally correlated with hemocyte dysfunction. After this time, the hemocytes recover, and encapsulation is then inhibited by other mechanism(s). In contrast, in parasitized tobacco hornworm larvae this type of inactivation in hemocytes of parasitized *M. sexta* larvae leads to irreversible cellular disruption. We have characterized the temporal pattern of expression of the CrV1-homolog from the *C. congregata* PDV in host fat body and hemocytes using Northern blots, and localized the protein in host hemocytes with polyclonal antibodies to CrV1 protein produced in *P. rapae* in response to expression of the CrV1 protein.

Host hemocytes stained with FITC-labeled phalloidin, which binds to filamentous actin, were used to observe hemocyte disruption in parasitized and virus-injected hosts and a comparison was made to hemocytes of nonparasitized control larvae. At 24 h post-parasitization host hemocytes were significantly altered compared to those of nonparasitized larvae. Hemocytes from newly parasitized hosts displayed blebbing, inhibition of spreading and adhesion, and overall cell disruption. A CrV1-homolog gene product was localized in host hemocytes using polyclonal CrV1 antibodies, suggesting that CrV1-like gene products of *C. congregata*'s bracovirus are responsible for the impaired immune response of the host.

Keywords: Insect immunity; Polydnavirus; Hemocyte; Encapsulation; Tobacco hornworm; Braconid wasp; Host immunosuppression

1. Introduction

The parasitoid wasp *Cotesia congregata* injects polydnavirus (CcPDV), venom, and 50–150 parasitoid eggs into its lepidopteran host *Manduca sexta* during the process of parasitization (Beckage et al., 1994; Alleyne and Beckage, 1997). Like the polydnavirus (PDV) of many braconid (bracoviruses or BVs) and ichneumonid (ichnoviruses or IVs) endoparasitoids, *C. congregata* and its PDV have evolved an intimate genetic relationship, and the genome of the virus is integrated in the chromosomal DNA of the wasp (Savary et al., 1999; Belle et al., 2002). Recently, the genome of CcBV has been sequenced in its entirety (Espagne et al., 2004), generating new insights into the CcBVs genomic organization and the evolution of PDVs. The primary

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doi:10.1016/j.jinsphys.2004.11.019

targets of these viruses appear to be the host immune and endocrine systems as seen in parasitized *M. sexta* larvae (Beckage, 1998; Dushay and Beckage, 1993; Beckage and Gelman, 2004).

The normal response of a non-habitual host insect to an invading metazoan parasite (i.e. parasitoid) is encapsulation. This process involves mobilization of host blood cells (granulocytes and plasmatocytes) to form a multi-layered capsule of hemocytes that engulf the invader (Luckhart and Webb, 1996; Harwood et al., 1998; Lavine and Strand, 2002). The parasitoid wasp(s) does not survive. In some host species, including *M. sexta* parasitized by *C. congregata* (Lavine and Beckage, 1995), hemocyte dysfunction occurs, dramatically reducing the number of functional hemocytes since the damaged cells are removed from circulation. In others, hemocytes alter their behavior, round up, and fail to spread over the surface of the substrate (i.e. a parasitoid egg) but effects are transitory and the hemocyte population eventually recovers, as occurs in *Pieris rapae* larvae parasitized by *Cotesia rubecula* (Asgari et al., 1996). The parasites nevertheless evade encapsulation despite recovery of host immunity, and successfully develop to maturity in the host hemocoel.

In *M. sexta* larvae parasitized by *C. congregata* several CcBV transcripts are detected beginning a few hours following wasp parasitization of the host (Harwood and Beckage, 1994; Harwood et al., 1998; Le et al., 2003). Virally encoded early protein 1 (EP1) is detected as soon as 30 min post-parasitization in the host's fat body and persists for several days (Harwood et al., 1994). At 24 h post-parasitization, PDV-encoded proteins have been shown to represent approximately 15% of host hemolymph proteins circulating in the hemolymph (Harwood and Beckage, 1994). In contrast, the *C. rubecula* PDV has only a few viral transcripts transiently expressed during a narrow temporal window between 4 and 8 h after parasitization (Asgari et al., 1996). The transcript CrV1 has been shown to be taken up by host hemocytes and causes their inactivation (Asgari and Schmidt, 2002). Other examples of temporary inactivation have been shown. In *Helicoverpa armigera* parasitized by the parasitoid *Campoletis chlorideae* host hemocytes are unable to mount a proper encapsulation response during the first four days of parasitism. After that point, host hemocytes regain their ability to encapsulate Sephadex beads (Yin et al., 2003) and, presumably, biotic targets. *Manduca sexta* larvae parasitized by *C. congregata* also exhibit inactivation of hemocytes, but in this case the dysfunction is permanent. The present study describes the effects of parasitization on host hemocytes which, in this system, are irreversibly altered in their appearance and are presumably rendered dysfunctional throughout development of the parasitoids in the host.

FITC-conjugated phalloidin was used to stain hemocytes, and the appearance of the cells was used as an indicator of the ability of host hemocytes to mount an immune response. Specifically, the FITC-conjugated phalloidin binds actin, allowing visualization of filamentous actin and the cytoskeleton of hemocytes. In addition, this allows a more detailed description of the morphological changes that occur upon inactivation of host hemocyte cells. A CrV1 probe was used to detect expression of CrV1-homolog mRNA and polyclonal CrV1-antibodies were used to detect expression of the CrV1 protein. Making a clear correlation between CrV1-homolog expression, inactivation of host hemocytes, and localization of the CrV1-homolog protein allows for further elucidation of the function of the CrV1-homolog in the *M. sexta*– *C. congregata* system.

2. Methods and materials

2.1. Insect rearing and fat body tissue collection

Insect stocks of the tobacco hornworm, *M. sexta*, and the braconid wasp parasitoid *C. congregata* were maintained as previously described (Beckage et al., 1994). Larvae were individually exposed to two wasp ovipositions as newly ecdysed fourth instars to ensure successful parasitization. Host larvae were parasitized on day 0 of the fourth instar and wasps emerged from the host on days 5 and 6 of the host's fifth instar. At predetermined time points larvae were ice-chilled and sacrificed to collect fat body of the host. Tissues were rinsed in 1X PBS (1.4 M NaCl, 20 mM KCL, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, ph 7.4) and frozen on dry ice while pooling host fat body tissue from several larvae of the same stage of development.

2.2. RNA isolation and blotting procedures

The techniques used for RNA preparation and blotting were as described in Le et al. (2003) and are briefly summarized here for convenience of the reader. Total RNA was isolated from tissues of parasitized and non-parasitized larvae using Trizol (GIBCO BRL, MD). Collected fat body tissue was homogenized with Trizol, incubated with chloroform at room temperature (RT), then centrifuged at 4 °C. The upper aqueous layer containing the RNA was precipitated with 75% ethanol and incubated at room temperature for 10 min. The mixture was centrifuged at 4 °C, and the resulting pellet was washed, dried, and resuspended in RNase-free water (EM Biosciences, IL). RNA quantification was spectrophotometrically determined using a Biotec GeneQuant RNA/DNA calculator II (Pharmacia, NJ). Aliquots of 15 μg/lane of total RNA from fat body tissue were electrophoresed in 1.2% formaldehyde

agarose gels at 100 V for 1.5 h using standard formaldehyde agarose gel electrophoresis protocols (Sambrook et al., 1989). The gel was blotted onto a nylon membrane using a VacuGene IL vacuum blotting system (Boehringer Mannheim, IN).

2.3. Synthesis of digoxygenin labeled probes for hybridization

The pBluescript KS II plasmid containing the CrV1 cDNA was used to prepare the CrV1 probe. The *M. sexta* S3 ribosomal probe (Jiang et al., 1996) contained in pBluescript SK was provided by Dr. Michael Kanost (Kansas State University, Kansas). Both plasmids were cloned in DH5-alpha *Esterechia coli* cells using TOPO cloning (Invitrogen) and minipreps were prepared using the Qiagen miniprep kit. The pBluescript KS II plasmid was cut with *Eco*RI and *Hind*III and electrophoresed on a 1% agarose gel, yielding a 1.2 kb CrV1 fragment. This fragment was cut out of the gel, purified using the Sephaglas BandPrep kit (Amersham Biosciences, NJ), and labeled using the digoxygenin High Prime DNA labeling and detection system (Roche Molecular Biochemicals). The same procedure was repeated for the S3 ribosomal probe, except the plasmid was cut with *Eco*RI and *Xho*I, yielding a fragment of ca. 800bp.

2.4. Northern hybridizations

The RNA blot was prehybridized and hybridized according to Digoxygenin Easy Hybridization protocols (Roche Molecular Biochemicals) using the CrV1 and S3 probe simultaneously. The mixture was allowed to hybridize overnight at 42 °C. After hybridization, the solution was removed, and the blot was washed twice with 2XSSC/0.1% sodium dodecyl sulfate at RT. The blot was then developed for chemiluminescence using CSPD (disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3,3.1.1$^{3.7}$]decan}-4-yl) phenyl phosphate) (Roche Molecular Biochemicals, Switzerland) and exposed to X-ray film (Fuji, CT) at RT for 15–30 min.

2.5. Actin staining in host hemocytes

Actin structure was visualized using a modification of a protocol previously developed for staining hemocytes of parasitized *Heliothis virescens* larvae (Webb and Luckhart, 1994). Larvae were bled from the prolegs into an ice-chilled 1.5 ml microcentrifuge tube. An aliquot of 15 μl of hemolymph was added to a glass slide with 35 μl of Grace's medium. Cells were allowed to settle for 15 min and the medium was removed from the slide and fresh Grace's medium was added. Cells were allowed to attach for 1 h in a humidified chamber, then washed twice with PBS. An aliquot of 35 μl of 4% paraformaldehyde was used to fix the cells for 15 min. Afterwards, PBS was used to wash the glass slide twice and 40 μl of 8–16 μg/μl FITC-conjugated phalloidin was applied to the glass slide. The hemocytes were then incubated for 1 h in the dark at RT. PBS was used to thoroughly wash the glass slides, and 1 drop of Vectashield (Vector Laboratories, CA) was applied and a cover-slip added. Cells were then visualized with a Nikon Eclipse fluorescence microscope fitted with a RS Photometrix digital camera.

2.6. CrV1-homolog uptake and antibody staining of hemocytes

CrV1-homolog uptake was assessed using a modified protocol of Asgari and Schmidt (2002). A larval proleg was severed and hemolymph was bled directly into an ice-chilled 1.5 ml microcentrifuge tube. An aliquot of 15 μl of hemolymph was added to a glass slide containing 35 μl of Grace's insect medium and incubated for 15 min in a humidified chamber. The solution was removed and fresh Grace's medium was added. Cells were allowed to attach to the glass slide for 1 h. Cells were washed with Tris-buffered saline Tween-20 (TBST) and fixed in 4% paraformaldehyde for 15 min. Cells were again washed twice with TBST. Anti-CrV1 polyclonal rabbit antibodies (1:500) were added to the glass slide and incubated for 1.5 h at room temperature. Cells were washed twice with TBST and incubated with FITC-conjugated anti-rabbit antibody (Sigma, NJ 1:100) in TBST-1% bovine serum albumin for 1 h at RT in the dark. Cells were washed several times with TBST and overlaid with Vectashield (Vector Laboratories, CA). Cells were then visualized with a Nikon Eclipse E800 fluorescence microscope as described above.

3. Results

Total RNA was extracted from fat body samples of naturally parasitized hosts at 4, 24, 48, 72 h post-parasitization and post wasp-emergence as shown in Fig. 1. The northern analysis using 15 μg of total RNA per lane and the CrV1 probe revealed the presence of a 1.4 kb fragment that was expressed beginning at 4 h post-parasitization and continued to be expressed throughout the course of parasitism. This 1.4 kb band was the expected size for the CrV1-homolog (Le et al., 2003). The transcript continued to be expressed throughout the course of parasitism, even when the wasps emerged from the host (Fig. 1), although expression levels of the CcV1 transcript appeared slightly lower, relative to ribosomal RNA expression, at wasp emergence. This may be indicative of a decline in expression when the parasitoids mature. CcV1 transcripts

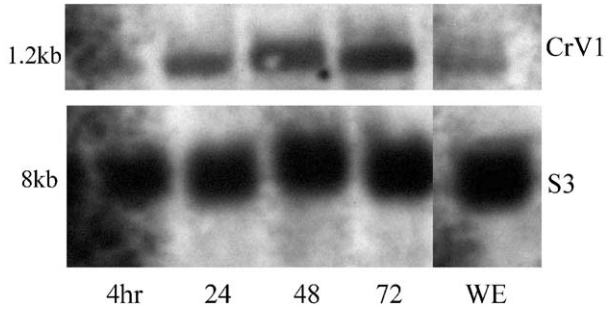

Fig. 1. A Northern blot showing the temporal expression of the CrV1-homolog in fat body tissues of parasitized *M. sexta* larvae. RNA was extracted from fat body tissue of parasitized fourth instar larvae at 4, 24, 48, 72 h post-parasitization and at wasp-emergence (WE). An aliquot of 15 μg of total RNA from each time point was electrophoresed on a 1.2% formaldehyde agarose gel, transferred to a nylon membrane, and probed with the digoxygenin-labeled CrV1 probe. A 1.2 kb band was detected in all samples. Levels of expression of S3 ribosomal RNA (Fig. 1B, bottom panel) were used as a control. Note that the post-WE signal of the CcV1 transcript appeared reduced relative to expression at earlier time points. [Figure reproduced from Le et al., 2003 with permission of Elsevier.]

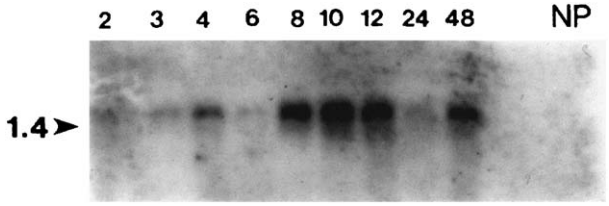

Fig. 2. Northern analysis of the temporal expression of the CrV1 homolog in hemocytes of parasitized hosts. Total RNA was extracted from hemocytes of parasitized larvae, and an aliquot of 8 μ RNA was loaded for each sample taken from 2 to 48 h post-parasitization and run on a formaldehyde gel. Following electrophoresis, northern blotting was carried out as described in Fig. 1 legend. The blot was probed with the Dig-labeled CrV1 probe, washed and developed using alkaline phosphatase. The blot was processed using 30 min exposure time to X-ray film. The 1.4 kb band seen in Fig. 1 was also present in hemocytes following parasitization at the time points examined up to 48 h post-parasitization. No signal was detected in hemocytes from nonparasitized larvae (NP). [Figure reproduced from Le et al., 2003 with permission of Elsevier.]

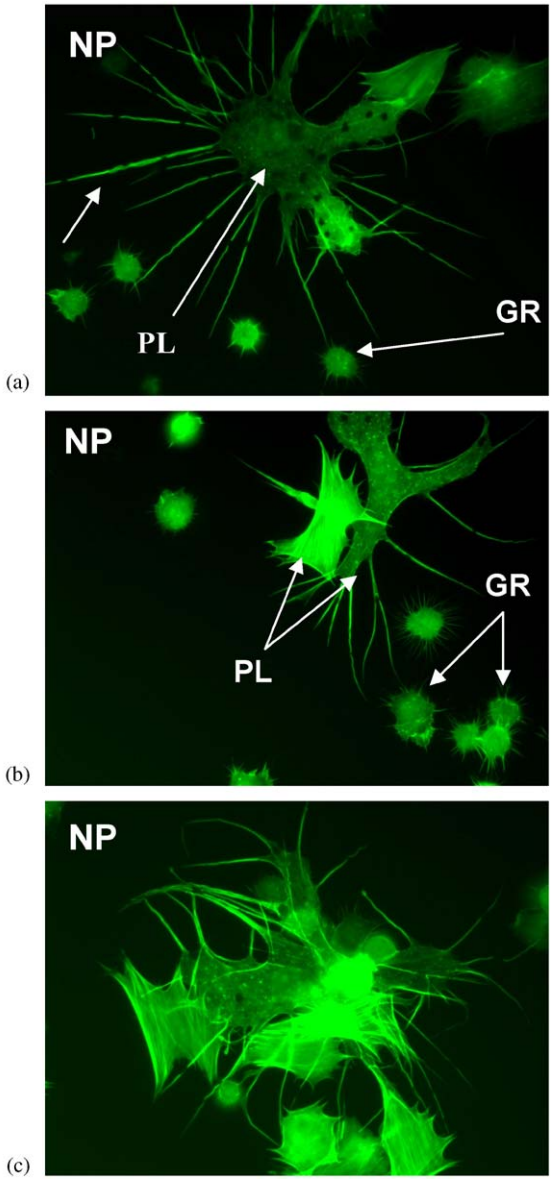

Fig. 3. Hemocytes of nonparasitized *M. sexta* stained with FITC-phalloidin. The appearance of plasmatocytes (PL) and granulocytes (GR) of non-parasitized larvae after they attach to the glass slide is shown. (a) It shows long filamentous filopodia particularly evident in the plasmatocytes (unlabeled arrow). Granulocytes displayed shorter filopodia with a "fried egg" appearance of attached cells. Plasmatocytes appeared to spread in cooperation with other cells, most often other plasmatocytes (b, c). This coordination presumably is indicative of an encapsulation response.

were similarly found in hemocytes from parasitized larvae when RNA extracts from the cells were analyzed in a Northern blot (Fig. 2).

Staining of hemocytes from parasitized *M. sexta* with FITC-conjugated phalloidin showed distinct differences in actin polymerization compared to actin-mediated assembly of the cytoskeleton in hemocytes of non-parasitized *M. sexta* larvae (Figs. 3 and 4). Hemocytes were observed at different time points post-parasitization. Because spreading behavior is a characteristic seen in healthy plasmatocytes and to a lesser extent in granulocytes, we sought to first analyze the behavior of spreading cells of non-parasitized control fourth instar larvae (Fig. 3a–c). The sample of control hemocytes had plasmatocytes with highly organized filopodia extending from the outer edges of the cell body inward towards the nucleus as the cells attached to the slide. The plasmatocytes were observed to extend their filopodia over the surface of the slide (Fig. 3a). Granulocytes had refractile granules in the cytoplasm and shorter filopodia extending from the outer surface of the cell (Fig. 3a, b). Plasmatocytes appeared to interact amongst themselves

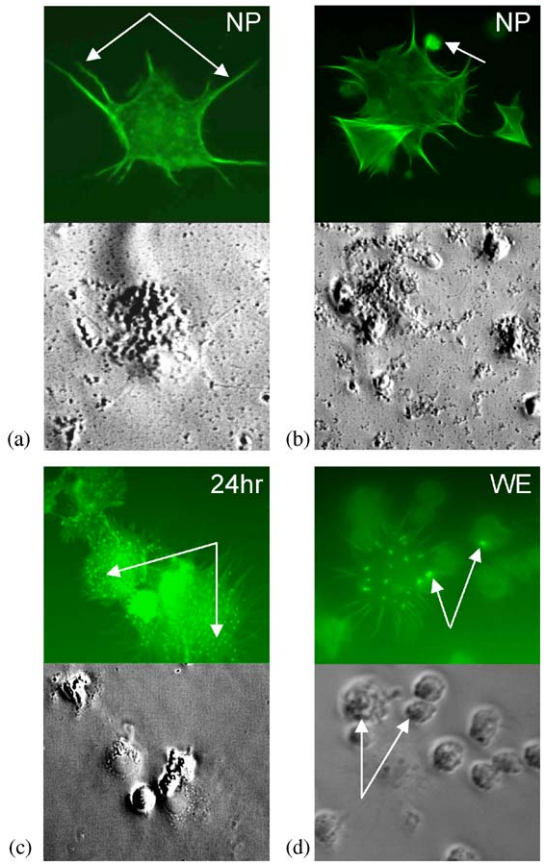

Fig. 4. Comparison of hemocytes from parasitized larvae at 24 h post-parasitization and WE to cells from non-parasitized *M. sexta* larvae stained with FITC-labeled phalloidin. Upper panels show actin staining under fluorescence microscopy and lower panels show light phase micrographs of the same field of cells. (a) It illustrates the intense staining of highly organized filopodia observed in plasmatocytes from nonparasitized *M. sexta* larvae. (b) It shows interacting plasmatocytes with highly organized filopodia and a single granulocyte (arrow). (c) It highlights the hot spots of concentrated phalloidin binding indicated by the numerous bright spots observed in either the cytoplasm or the surface of the spread hemocytes. This characteristic is also highlighted by arrows in (d), showing intense spots of increased phalloidin binding. Note cells in (d) are blebbing (bottom panel, arrows).

(Fig. 3b, c) and interdigitate the filopodia of adjacent cells in a representative encapsulation response as the cells attached to the slide.

Several distinct alterations were observed in hemocytes from parasitized larvae as compared to hemocytes from non-parasitized larvae (Fig. 4a–d). By 24 h post-parasitization there was a visible change in the morphology of the hemocyte cells when stained actin was viewed (Fig. 4c, upper panel). 'Hot spots' of intense staining of phalloidin binding sites were observed in hemocytes examined 24 h post-parasitization (Fig. 4c) and also later on the day of wasp emergence (Fig. 4d, upper panel, arrows). Cells from parasitized *M. sexta* had less numerous and shorter filopodia extending from the outer edges of the hemocytes compared to hemocytes from nonparasitized larvae. Hemocytes from parasitized larvae appeared to clump together and coalesce into aggregates that appeared to contain hemocytes that were blebbing.

To investigate whether the period of hemocyte inactivation is associated with production of the CrV1-like homolog protein, antibody staining experiments were carried out to localize expression of the CrV1-homolog protein (Fig. 5a–d). This allowed determination of whether the CrV1-homolog in *C. congregata* has a similar function to the CrV1 gene in *C. rubecula*, and is taken up by host hemocytes. Fig. 5b–d illustrate the time course progression of CrV1-like protein detection in host hemocytes following parasitization by *C. congregata*. The CrV1-like protein is detected as early as 24 h (Fig. 5b) after parasitization and persists throughout the course of parasitism until the wasps emerge (Fig. 5c, d). This temporal pattern is directly supported by the expression of the CcV1 transcript in host fat body (Fig. 1) and hemocytes (Fig. 2) and the sustained altered behavior of host hemocytes observed through staining with FITC-conjugated phalloidin (Fig. 4).

4. Discussion

This study documented the pattern of the temporal expression of the CrV1-homolog transcript in host fat body tissues (Fig. 1) and hemocytes (Fig. 2). We characterized changes in hemocyte behavior induced by parasitism (Fig. 4) and localized the CrV1-like protein within the hemocytes of the host (Fig. 5). CrV1 is one of few viral transcripts expressed by the *C. rubecula* PDV in host *P. rapae* larvae (Asgari et al., 1996; Glatz, 2004). Its expression occurs transiently between 4 and 8 h after parasitization and causes temporary inactivation of host hemocytes. Asgari et al. (1996) showed that during the period of hemocyte inactivation the CrV1 mRNA was expressed and the protein was detected in the hemocyte cells of the host.

This project characterized the temporal expression of the CrV1-homolog transcript throughout the course of parasitism in host fat body tissues and showed that it was detected from 4 h post-parasitization and persisted until the wasps emerged from the host (Fig. 1). At time points up to 48 h post-parasitization we also detected CcV1 in host hemocytes (Fig. 2). In addition, suppression of the immune system was observed in *M. sexta* larvae parasitized by *C. congregata*. Using FITC-conjugated phalloidin it was observed that as early as 24 h after parasitization a change in the encapsulation response by host hemocytes occurs. In comparing hemocytes from nonparasitized and parasitized larvae using fluorescence microscopy, it was observed that by 24 h post-parasitization the hemocytes had experienced significant morphological changes. The encapsulation

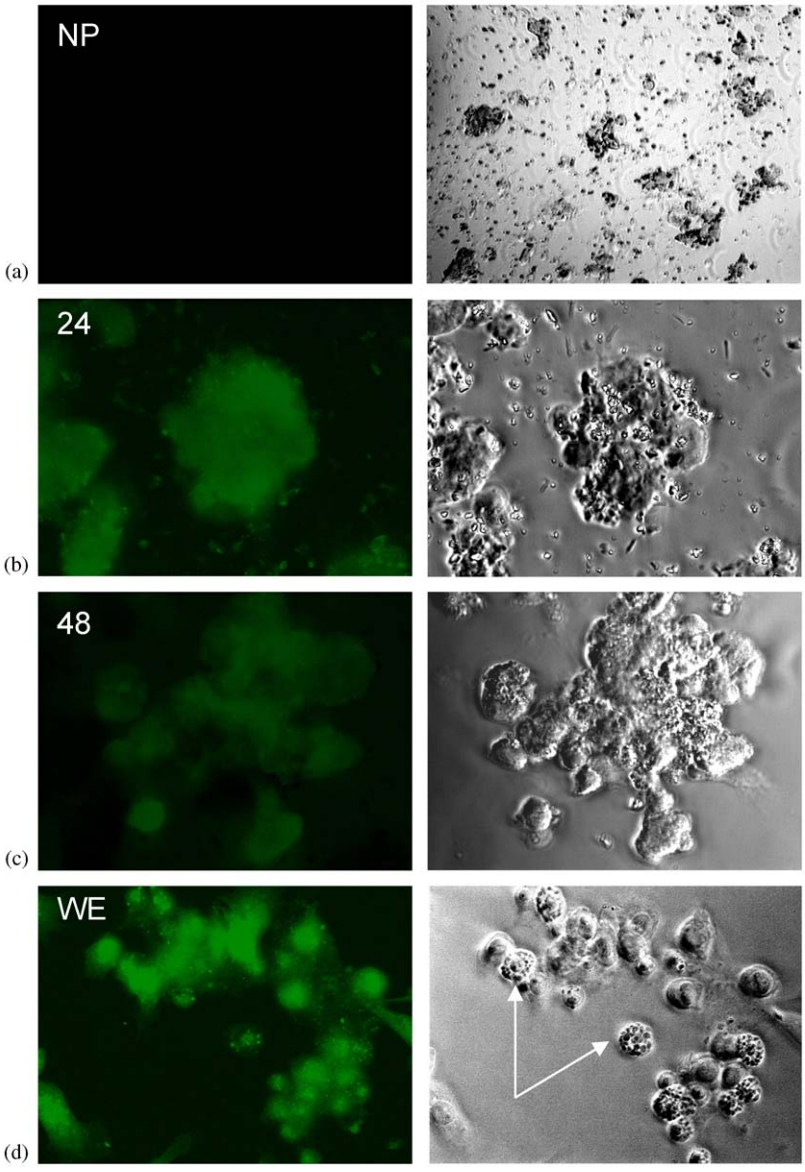

Fig. 5. Time course progression of CcV1 protein detection in hemocytes of parasitized *M. sexta* larvae. The left panels show CrV1 antibody binding to epitopes in the cells from parasitized larvae and the right panels are light phase micrographs of the same fields of cells. The CrV1-like protein detection in hemocytes from a nonparasitized control larva (NP) (a) is compared to larvae at 24 and 48 h post-parasitization (b and c, respectively) and at WE (d). Overall the level of protein expression and signal intensity were greatest at 24 h post-parasitization and WE. Arrows on the right panel of (d) highlight blebbing cells from hosts with emerged wasps.

response of non-parasitized host hemocytes was observed, and we noted their ability to adhere and spread as they attached to the glass slide (Fig. 3). It also appears that the ability of cells to act cooperatively and form multicellular aggregates with one another is also an indicator of whether the immune system is operating properly. This was most evident in Fig. 3c showing cells that were closely attached to one another in hemolymph taken from nonparasitized larvae.

At 4 h post-parasitization, changes in host hemocyte morphology were not yet evident (data not shown) but by 24 h dramatic morphological changes were apparent in the cells (Fig. 4). Large clumps of dead and dying cells were found in hemolymph from parasitized larvae. The link between parasitization and the inability of hemocytes to attach and spread has been demonstrated in some other host-parasite systems (Lavine and Strand, 2002) in addition to *M. sexta–C. congregata* (Lavine and Beckage, 1995). Newly parasitized fourth instar larvae of *M. sexta* fail to encapsulate abiotic targets such as Sephadex beads (Lavine and Beckage, 1996). As seen in Fig. 3, hemocytes from non-parasitized larvae have a highly organized cytoskeleton in contrast to the clumps of dysfunctional hemocytes seen in parasitized larvae.

The cells surrounding the large central cells of the clumps appeared to be dead or dying, as indicated by the presence of blebs on the outer surface of the cells (Fig. 5d, right panel). Using FITC-labeled phalloidin allowed for earlier detection of morphological changes in host hemocytes that affected cooperation between one another during the encapsulation response and formation of the multicellular layer of cells. Similar disruption of the cytoskeleton of host hemocytes has been seen in *Lacanobia oleracea* parasitized by the parasitoid *Eulophus pennicornis* (Richards and Edwards, 2002). Interestingly we have observed that in hemolymph the overall number of hemocytes in circulation decreases 3-fold as early as 24 h following parasitization. In contrast *L. oleracea* parasitized by *E. pennicornis* hemocytes numbers increase up to day three post-parasitization and then the hemocyte count begins to decline (Richards and Edwards, 1999). The reasons accounting for these differences between species are unknown.

In parasitized *P. rapae* larvae CrV1 protein is taken up by host hemocytes following its synthesis and secretion by the cells (Asgari and Schmidt, 2002). Presence of the protein in the hemocytes alters their behavior. Hemocytes from parasitized *L. oleracea* larvae have extra proteins detectable in the cells in addition to those found in hemocytes from non-parasitized larvae. In our study of parasitized *M. sexta*, the CcV1 mRNA was detected in fat body tissues throughout the course of parasitism (Fig. 1). Hemocytes also showed that CcV1 is expressed in those cells for up to 48 h post-parasitization, the latest time point tested (Fig. 2). Staining the hemocytes with FITC-labeled phalloidin revealed major differences in the cytoskeletal architecture in cells from parasitized versus nonparasitized larvae (Fig. 4). We observed binding of CrV1 polyclonal antibodies to antigens present in/on the cells (Fig. 5). At this point it is unclear if binding of the antibodies to hemocyte antigens is confined to the cell surface or is distributed throughout the cytoplasm as suggested by the images seen in Fig. 5. Blebbing is evident in cells from parasitized larvae, particularly in hemocytes examined at the time of emergence of the wasps from the host (Fig. 5d, right panel). Although whether the blebbing is preparatory to the induction of apoptotic events in the cells is not yet known, but this possibility appears likely based upon the observed morphological changes in the cells. These observations merit additional experiments using caspase assays or TUNEL labeling to confirm on a molecular level that these changes signal the onset of the apoptotic pathway in the cells.

Further studies should help elucidate the function and *modus operandi* of the PDV gene products expressed in parasitized *M. sexta* larvae. Asgari and Schmidt (2002) showed that CrV1 protein encoded by the PDV of *C. rubecula* required a coiled-coil domain containing a putative leucine zipper for proper CrV1 function which was required for induction of hemocyte inactivation in the *P. rapae* host. Without the coiled-coil domain the protein was not taken up by host hemocytes (Asgari and Schmidt, 2002). It remains to be seen whether the CrV1-homolog in *C. congregata* PDV contains a similar coiled-coil domain which facilitates entry of the virally encoded protein into target cells such as fat body or hemocytes following parasitization the hornworm host.

Acknowledgements

The authors thank Dr. Michael Kanost (Kansas State University) for providing the S3 ribosomal RNA probe. We also thank Dr. Prue Talbot (University of California-Riverside) and Ms. Jennifer Lenz (Beckage lab) who provided insightful comments and editorial assistance with the manuscript. The research was conducted using funding awarded by the UCR Environmental Toxicology Graduate Program and a Sigma Xi grant-in-aid awarded to KEA, and a University of California UC-Mexus grant to NEB.

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