Effects of Parasitization and Envenomation by the Endoparasitic Wasp *Pimpla turionellae* (Hymenoptera: Ichneumonidae) on Hemocyte Numbers, Morphology, and Viability of Its Host *Galleria mellonella* (Lepidoptera: Pyralidae)

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ABSTRACT Venom from the pupal endoparasitoid *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae) contains a mixture of biologically active components, which display potent paralytic, cytotoxic, and cytolytic effects toward hosts. Here, we further investigate whether parasitism or envenomation by *P. turionellae* alters hemocyte numbers of its host *Galleria mellonella* L. (Lepidoptera: Pyralidae). Total hemocyte counts declined sharply in pupae and larvae of *G. mellonella* exposed to *P. turionellae*. These same cellular responses occurred when wasp venom was artificially injected into hosts, suggesting that venom alone induces cytotoxicity in hemocytes. Analysis of the differential hemocyte counts in untreated pupae and larvae revealed that more than half of the circulating hemocytes were granular cells followed by plasmatocytes. Parasitism reduced the number of granular cells while increasing the number of plasmatocytes. This trend was most evident at 4 h postparasitism, and a similar trend was observed with the artificial injection of high (but not low) doses of venom. When isolated larval hemocytes were exposed to a LC_{99} dose of venom, a differential response was observed for granular cells versus plasmatocytes. Both types of cells displayed some formation of vacuoles within the cytoplasm within 15 min posttreatment. However, the degree of vacuole formation was much more extensive in granular cells at later time points than for plasmatocytes, and granular cells seemed much more susceptible to venom as evidenced by cell death.

KEY WORDS cytotoxicity, hemocyte count, hemocyte viability, wasp venom

Parasitic wasps alter a variety of biochemical and physiological processes in their hosts to create a more favorable environment for the parasitoid's progeny. Endoparasitic wasps deposit their eggs in the body cavity of the insect host, which thus requires a suppression of the host immune/defense system to ensure survival. In most cases, adult females inject factors (e.g., ovarian proteins, virus-like particles, polydnaviruses [PDVs] and/or venom) of maternal origin during oviposition that adversely affects host hemocyte numbers and behavior and alters expression/release of recognition factors that "recruit" hemocytes for wound repair and/or encapsulation of foreign bodies (Kitano 1986; Tanaka 1987; Genedani et al. 1994; Ottaviani et al. 1995; Glatz et al. 2004a,b; Zhang et al. 2004; Morales et al. 2005). However, the role of venom from endoparasitoids in disabling host hemocytes is poorly understood and has received considerably little study by comparison to other maternally derived secretions (Moreau and Guillot 2005, Asgari 2007). Mochiah et al. (2003) reported that the immune system of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) was suppressed (decreased hemocyte count) when parasitized by *Cotesia sesamiae* (Cameron) (Hymenoptera: Braconidae), but venom alone could not evoke this immunological suppression. Calyx fluids from *C. plutellae* (Kurdjumov) (Hymenoptera: Braconidae) have been shown to play a major role in the suppression of the host immune system, and wasp venom is involved, but because it has only a limited effect on hemocytes, the venom's action probably synergizes the effect of calyx fluid or PDVs (Yu et al. 2007). Venoms from these koinobiont species are frequently associated with temporary paralysis (Gauld 1988) and in most of the cases enhance the effects of PDVs or calyx fluid rather than serving as separate immunological suppressants (Davies et al. 1987, Tanaka 1987, Gupta and Ferkovich 1998, Beckage and Gelman 2004).

In contrast to koinobiontic parasitoids, most of the idiobiont species paralyze their hosts permanently, essentially preserving the hosts while the parasitoid progeny feed and develop (Wharton 1993). Generally, the hosts of idiobionts are thought to be passive

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containers of nutrients because they have been paralyzed or killed rapidly by the parasitoids, and presumably cannot mount a defensive response to host attack (Schmidt et al. 2001). Thus, no suppression of host defenses would be warranted. Consistent with this view, the role of idiobiont endoparasitoid venom and/or parasitism in suppressing host immune defense and their effects on the hemolymph profile and hemocytes of hosts have not been studied in any detail. However, a limited number of studies suggest that venom from idiobiont endoparasitoids devoid of symbiotic viruses may alone perturb host immune defenses. For example, venom from *Pimpla hypochondriaca* (Retzius) (Hymenoptera: Ichneumonidae) has been shown to be cytotoxic to hemocytes from host species and has a potent anti-hemocyte action that can impair hemocyte-mediated immune responses (Richards and Parkinson 2000). More recently, Cai et al. (2004) reported that parasitism of *Pieris rapae* L. (Lepidoptera: Pieridae) by the pupal endoparasitoid *Pteromalus puparum* L. (Hymenoptera: Pteromalidae) induced changes in host hemocyte number and behavior resulting from the activity of wasp venom.

The solitary idiobiont pupal endoparasitoid *P. turionellae* produces venom that contains a number of biologically active components including melittin, apamin, the biogenic amines histamine and serotonin, and the catecholamine noradrenaline. In addition, venom from this endoparasitoid wasp contains several mid- to high-range molecular weight proteins (Uçkan et al. 2004, Uçkan et al. 2006, Ergin et al. 2007) and displays potent paralytic, cytotoxic, and cytolytic effects toward lepidopteran and dipteran hosts (Ergin et al. 2006). The details of how the venom operates to induce host paralysis and evoke cell death have also been partially determined in previous studies (Keenan et al. 2007, Rivers et al. 2007). An inhibition of hemocyte pseudopodia formation and encapsulation by venom from *P. turionellae* has been reported previously (Osman 1978). The current study was undertaken to investigate further whether *P. turionellae* parasitism and/or venom affect the total and differential hemocyte numbers of its host *G. mellonella.* Venom-induced changes in cell morphology were also examined using in vitro assays with hemocytes from *G. mellonella.*

Materials and Methods

Parasitoid and Host Rearing. *P. turionellae* were reared on the pupae of the host, *G. mellonella* at 25 ± 1°C, 60 ± 5% RH, and with a photoperiod of 12:12 (L:D) h. Adult parasitoids were fed 30% (vol:vol) honey solution and provided with host pupae (four pupae for every 10 female wasps once every 3 d). Host colony was maintained by feeding the insects with honeycomb.

Preparation of *P. turionellae* Venom and Injection Into *G. mellonella.* Venom reservoir contents were isolated from honey- and host-fed 15–20-d-old females as described previously (Uçkan et al. 2004). The isolated crude venom was adjusted to doses below the LD_{99} calculated for *G. mellonella* pupae and larvae (Ergin et al. 2006). To do so, venom was adjusted to 0.05, 0.02, 0.01, and 0.005 venom reservoir equivalents (VREs) for pupae and 0.5, 0.1, 0.05, and 0.02 VREs for larvae with phosphate-buffered saline (PBS; 0.138 M NaCl and 0.0027 M KCl in 0.01 M PBS, pH 7.4) after centrifuging at 3,000 × *g* at room temperature for 10 min to remove cell debris. A 5-μl solution of the venom preparation was injected between the last two lateral abdominal segments of 1–2-d-old host pupae (140 ± 20 mg) and on the first hind leg of last instar larvae (260 ± 10 mg) by using a 10-μl Hamilton microsyringe (Hamilton Co., Reno, NV). Petroleum jelly was applied to the injection area to prevent hemolymph loss. Controls consisted of pupae and larvae untreated, null-injected and injected with only 5 μl of PBS.

Parasitization of *G. mellonella* Pupae. Parasitization was performed on 1- or 2-d-old host pupae by exposing an individual host pupa (140 ± 20 mg) to an individual 15–20-d-old wasp female. Parasitized pupae were held at 25 ± 2°C, 60 ± 5% RH under a photoperiod of 12:12 (L:D) h, as were the controls and venom-treated pupae, until hemolymph collection. *P. turionellae* females normally parasitize host prepupae and pupae in nature (Kansu and Uğur 1984); therefore, parasitization was not used as an experimental assay for larvae of *G. mellonellae.*

Hemolymph Collection, Total and Differential Hemocyte Counts. To investigate the effects of parasitization or venom injection on the number of circulating hemocytes 1–2-d-old host pupae and last instars of approximately the same sizes were used. Pupae were bled by piercing the cuticle at the abdomen and larvae on the first hind leg with a sterile 19-gauge needle. Four microliters of hemolymph from each individual pupa and larva were collected with a glass microcapillary tube (Sigma, St. Louis, MO). To avoid hemocyte aggregation, hemolymph from pupae and larvae was immediately mixed to 20 and 36 μl with ice cold anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM Na_2 EDTA, and 41 mM citric acid, pH 4.5) in sterile Eppendorf tubes, respectively. Ten microliters of hemolymph suspension diluted in anticoagulant solution were applied to an improved Neubauer hemocytometer (Superior, Bad Mergentheim, Germany) after gently mixing by passing through a micropipette.

Differential hemocyte counts were also assayed by piercing the pupae and larvae at the same site and with the same method given above and immediately spreading the hemolymph without permitting melanization (mixed with the anticoagulant buffer) onto sterile microscopic slides. The slides were allowed to air dry for 20–30 min to facilitate the adhesion of hemocytes to the glass. Cells were then fixed in methanol for 5 min. Once the fixative had evaporated, hemocytes were stained with Giemsa (3 ml of Merck Giemsa's azure eosine methylene blue solution in 57 ml of PBS, pH 7.4) for 10 min, and slides were rapidly washed with distilled water and PBS and then air-dried. Total and differential hemocyte counts (total hemocyte count [THC] and differential hemocyte

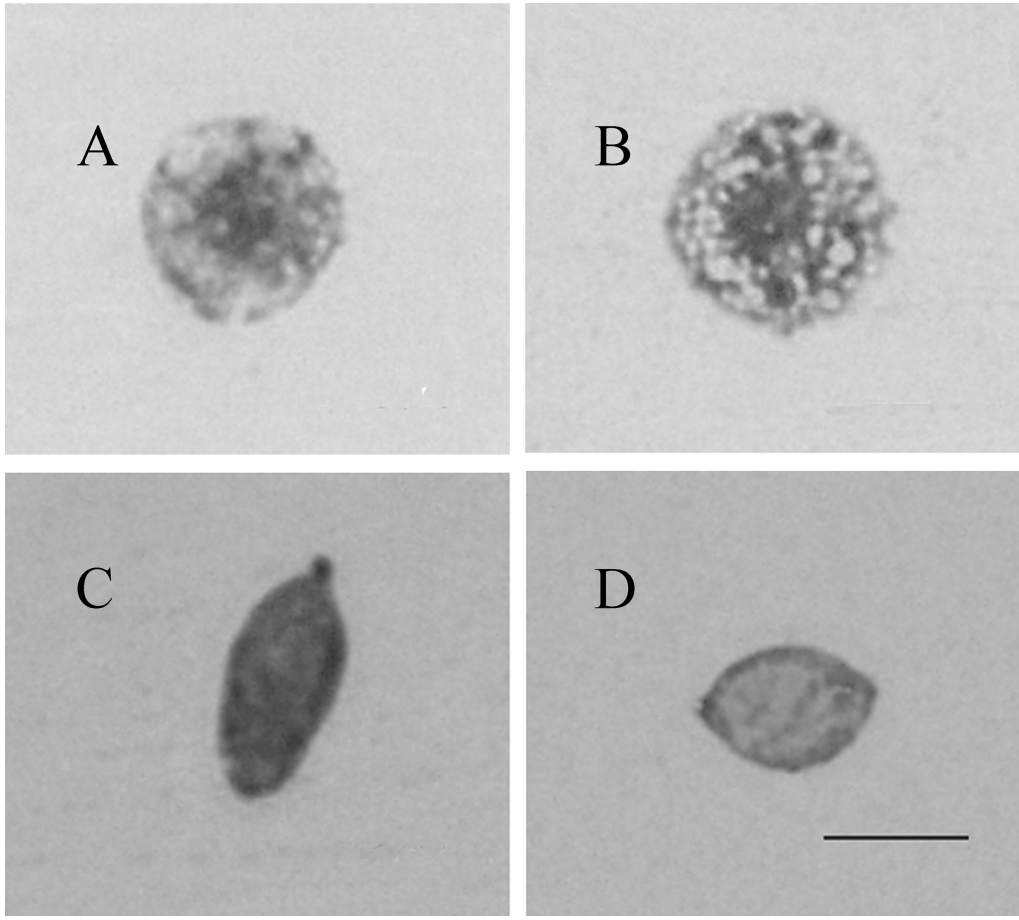

Fig. 1. Granulocytes from last instars (A) and pupae (B) and plasmatocytes from last instars (C) and pupae (D) of *G. mellonella.* Photographs were made using a 1,000× objective. Bars = 10 μm.

count [DHC]) were assayed at three time intervals (4, 8, and 24 h posttreatment) under a BX51 microscope (Olympus, Tokyo, Japan) with bright field and phase contrast optics. Differential hemocyte counts were performed and expressed as the relative number of different types of hemocytes per total 1,000 hemocytes counted at a given time point.

Hemocyte types were classified by morphological characteristics (Rowley and Ratcliffe 1981, Gupta 1985, Ribeiro and Brehelin 2006). Granular cells were round cells with granules in their cytoplasm, whereas plasmatocytes assumed a spindle shape in the unspread state and upon spreading they exhibited a bilateral cytoplasmic extension (Fig. 1). Other types of hemocytes observed were collectively named as "others" in this study. Controls consisted of pupae and larvae untreated, null-injected (injection was done by an empty microsyringe) and injected with only 5 μl of PBS. Five host pupae and larvae were evaluated for each experimental and control assays in three replicates at a given time for total hemocyte counts, whereas 1,000 cells from an individual pupa and larva were counted and differentiated in each of four replicates for differential hemocyte counts.

Hemocyte Assays. Venom-induced changes in cell morphology were examined using an in vitro assay with isolated hemocytes. Hemocytes were collected as described above from last instars of *G. mellonella.* After centrifugation (3,000 × *g* for 10 min at room temperature) in anticoagulant buffer, hemocytes were resuspended in TC-100 insect media containing 10% fetal bovine serum (FBS). Then, 2 μl of cell suspension (containing ≈2,000 cells) was transferred to 100 μl of culture media in each well of a 96-well plate (Falcon; BD Biosciences Discovery Labware, Bedford, MA) and allowed to incubate at 27°C for 1 h. Hemocytes were tested by using saline (venom isolation buffer) or venom only (LC_{99} dose = 0.001 VRE/μl), in 1-μl volumes added to hemocyte monolayer. Changes in cell morphology were monitored using an Insight four SPOT RT fire wire digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) mounted on a phase contrast inverted microscope (Eclipse TE-300, Nikon, Tokyo, Japan) and connected to Macintosh Power Mac G5 computer (Apple Computer, Cupertino, CA). Observations of adhesion and spreading, as well as hemocyte viability were made at

Table 1. Total hemocyte counts (× 10^6 cell/ml) of *G. mellonella* pupae experimentally envenomated and parasitized by *P. turionellae*

Treatment	Total hemocyte count (× 10^6 cell/ml) (mean ± SEM)[a] Time posttreatment (h)[b]			Statistics (ANOVA)		
	4	8	24	*F*	df	*P*
Untreated	10.60 ± 1.5a x	12.00 ± 1.1a x	10.14 ± 1.1a x	0.601	2, 42	0.553
Null-injected	6.57 ± 1.3b x	7.98 ± 1.1b x	7.17 ± 0.9ab x	0.395	2, 42	0.676
PBS-injected	3.99 ± 0.6b x	3.29 ± 0.5c x	6.73 ± 0.5bc y	11.829	2, 42	0.000
0.005 VRE-injected	3.82 ± 0.5b x	4.87 ± 0.6c x	4.67 ± 0.6bcd x	0.996	2, 42	0.378
0.01 VRE-injected	4.07 ± 0.7b x	3.37 ± 0.5c x	5.26 ± 0.7bcd x	2.243	2, 42	0.119
0.02 VRE-injected	4.12 ± 0.5b x	3.39 ± 0.6c x	3.83 ± 0.5cd x	0.451	2, 42	0.640
0.05 VRE-injected	4.02 ± 0.9b x	2.85 ± 0.4c x	2.48 ± 0.2d x	1.805	2, 42	0.177
Parasitized	4.32 ± 0.6b x	4.32 ± 0.6c x	4.57 ± 0.6bcd x	0.058	2, 42	0.944

[a] Each represents the mean of hemocyte counts from 15 pupae (140 ± 20 mg).
[b] Numbers in columns (a–d) and rows (x–y) followed by the same letter are not significantly different ($P > 0.05$).

15-min intervals for 3 h and then at 3-h intervals for 24 h.

Statistical Analysis. Means were compared using one- and two-way analysis of variance (ANOVA) and subsequently, means were separated using Tukey's honestly significant difference (HSD) post hoc test, or Student–Newman–Keuls multiple comparisons tests. Data were analyzed either with SPSS version 10.0 for Windows (SPSS Inc. 1999) or StatView version 5.01, (Abacus Concepts, Berkley, CA). Results were considered statistically significant when $P < 0.05$.

Results

Effects of Parasitization and Venom Injections on THC. Hemolymph samples from untreated pupae normally displayed 10.60, 12.00, and 10.14 × 10^6 cells/ml at 4, 8, and 24 h posttreatment, respectively. Hemocyte number of *G. mellonella* pupae differed significantly among treatments at 4 h ($F = 6.614$; df = 7, 112; $P = 0.000$), 8 h ($F = 19.972$; df = 7, 112; $P = 0.000$), and 24 h ($F = 11.864$; df = 7, 112; $P = 0.000$) after treatment (Table 1). THC indicated a considerable decline in the number of circulating hemocytes in *G. mellonella* pupae exposed to *P. turionellae* or any dose of wasp venom injected experimentally. The number of hemocytes per milliliter in the parasitized and venom-injected group decreased >50%, regardless of time and venom dose (Table 1). The minimum count of 2.48 × 10^6 cells/ml was observed at 24 h after venom injection at the highest dose of 0.05 VRE injected, whereas a maximum of 5.26 × 10^6 cells/ml were determined at 0.01 VRE. The effects of null-injection and PBS-injection were more consistent with parasitized and venom-treated groups than that of untreated groups. It was evident that both treatments differed from untreated control pupae at four and 8 h posttreatments, whereas the hemocyte number per ml remained high in null-injection at 8 h (7.98 × 10^6 cells/ml). However, at 24 h no significant difference was observed between null-/PBS-injected and parasitized and venom-treated groups except for the highest venom dose tested (0.05 VRE). The effect of venom injection and parasitization on the hemocyte number of host pupae was treatment dependent ($P = 0.000$) but not time ($P = 0.517$) dependent, and the relationship between treatment and the hemocyte number was not influenced by time ($P = 0.109$) (Table 2).

In contrast, the number of hemocytes differed significantly with respect to the type of treatment ($P = 0.000$) and time ($P = 0.000$), and the relationship between treatment and the hemocyte number was influenced by time ($P = 0.021$) in host larvae (Table 2). Untreated larvae typically displayed 15.77, 17.51, and 17.49 × 10^6 cells/ml at 4, 8, and 24 h posttreatments, respectively. Hemocyte number differed significantly among treatments at 4 h ($F = 4.967$; df = 6, 98; $P = 0.000$), 8 h ($F = 6.283$; df = 6, 98; $P = 0.000$), and 24 h ($F = 13.726$; df = 6, 98; $P = 0.000$) after treatment (Table 3). THC did not indicate a considerable change in null-, PBS- or low doses of venom-injected (0.02 and 0.05 VREs) pupae. However, the number of hemocytes per ml significantly decreased in larvae injected with a 0.1 VRE dose at 24 h posttreatment. The minimum numbers of hemocytes were observed at 8 and 24 h posttreatment as 9.61 × 10^6 and 6.21 × 10^6 cells/ml when pupae were injected with the highest dose of venom 0.5 VRE (Table 3).

Effects of Parasitization and Venom Injections on DHC. To determine whether experimental venom injection and parasitism (only for pupae) of *P. turionellae* alter the hemocyte population of *G. mellonella*, both pupae and larvae were bled after parasitism or injections at different time intervals posttreatment. Morphologically, the hemocytes of *G. mellonella* were classified into granular cells, plasmatocytes, and other types. Granular cells were round-shaped and possessed granules in the cytoplasm whereas plasmato-

Table 2. ANOVAs of the effects of different treatments, time, and their interactions on the total hemocyte count of *G. mellonella*

Stage	Source	df	MS	*F*	*P*	r^2
Pupa	Treatment	7	291.833	32.052	0.000	0.42
	Time	2	6.020	0.661	0.517	
	Treatment × time	14	13.657	1.500	0.109	
	Error	336	9.105			
Larva	Treatment	6	556.958	17.767	0.000	0.42
	Time	2	295.982	9.442	0.000	
	Treatment × time	12	64.121	2.045	0.021	
	Error	294	31.347			

Table 3. Total hemocyte counts (× 10^6 cell/ml) of *G. mellonella* larvae experimentally envenomated by *P. turionellae*

Treatment	Total hemocyte count (× 10^6 cell/ml) (mean ± SEM)[a]			Statistics (ANOVA)		
	Time posttreatment (h)[b]					
	4	8	24	*F*	df	*P*
Untreated	15.77 ± 1.8ab x	17.51 ± 1.6a x	17.49 ± 1.1a x	0.444	2, 42	0.644
Null-injected	21.87 ± 3.0a x	19.79 ± 1.2a x	15.31 ± 1.1a x	2.992	2, 42	0.061
PBS-injected	21.79 ± 1.3a x	15.83 ± 1.8a y	16.04 ± 1.6a y	4.653	2, 42	0.015
0.02 VRE-injected	18.27 ± 0.9ab x	18.19 ± 0.9a x	18.57 ± 1.1a x	0.045	2, 42	0.957
0.05 VRE-injected	18.73 ± 1.7ab x	18.28 ± 1.4a x	14.67 ± 1.6ab x	2.046	2, 42	0.142
0.1 VRE-injected	12.16 ± 0.9b xy	14.77 ± 1.3ab x	10.24 ± 0.9bc y	4.717	2, 42	0.014
0.5 VRE-injected	12.99 ± 1.9b x	9.61 ± 1.1b xy	6.21 ± 0.6c y	6.627	2, 42	0.003

[a] Each represents the mean of hemocyte counts from 15 larvae (260 ± 10 mg).
[b] Numbers in columns (a–c) and rows (x–y) followed by the same letter are not significantly different ($P > 0.05$).

cytes were spindle-shaped (Fig. 1). There were also prohemocytes and spherulocytes that were collectively named as others (OT).

In *G. mellonella* pupae, the major hemocyte type was the granular cells that comprised 56.08 ± 3.18, 58.60 ± 0.98, and 57.60 ± 0.99% in the total hemocyte population of untreated individuals at three time intervals (4, 8, and 24 h posttreatment). Plasmatocytes were the next most frequent hemocyte type and represented 42.80 ± 0.30, 40.40 ± 0.98, and 41.30 ± 0.84% of the total numbers of hemocytes, respectively (Table 4). Only parasitized pupae displayed a significantly lower number of granulocytes at 4 h ($F = 4.615$; df = 7, 24; $P = 0.002$) compared with counts taken from controls and venom-injected insects. In *G. mellonella* pupae injected with higher doses of venom (0.02 and 0.05 VREs), granular cells accounted for <50% of the total hemocyte population, further decreased to ≈37% in parasitized pupae, whereas this ratio was 56% in untreated individuals. However, no significant difference was observed among treatments in granular cells counts at 8 h ($F = 3.157$; df = 7, 24; $P = 0.058$) and 24 h ($F = 2.065$; df = 7, 24; $P = 0.088$) (Table 4). In contrast, the ratio of plasmatocytes was significantly higher ($F = 4.600$; df = 7, 24; $P = 0.002$) in pupae parasitized by wasps at 4 h posttreatment compared with the controls and lower venom doses (0.005 and 0.01 VREs). A significant variation in the number of plasmatocytes among treatments also appeared at 8 h ($F = 3.662$; df = 7, 24; $P = 0.008$), whereas no significant

Table 4. Differential hemocyte counts (cells/1,000) of *G. mellonella* pupae experimentally envenomated or parasitized by *P. turionellae*

Hemocyte type	Treatment	Differential hemocyte count (cells/1,000) (mean ± SEM)[a]			Statistics (ANOVA)		
		Time posttreatment (h)[b]					
		4	8	24	*F*	df	*P*
GR	Untreated	560.75 ± 31.8a x	586.00 ± 9.8a x	576.00 ± 9.9a x	0.403	2, 9	0.680
	Null-injected	531.25 ± 18.4a x	545.25 ± 21.1a x	513.25 ± 17.1a x	0.715	2, 9	0.515
	PBS-injected	613.75 ± 26.6a x	583.25 ± 59.2a x	520.00 ± 29.0a x	1.360	2, 9	0.305
	0.005 VRE-injected	535.25 ± 13.4a x	509.75 ± 5.4a x	532.75 ± 15.0a x	1.362	2, 9	0.304
	0.01 VRE-injected	566.25 ± 16.0a x	551.25 ± 10.8a xy	461.25 ± 41.2a y	4.668	2, 9	0.041
	0.02 VRE-injected	492.50 ± 8.3ab x	464.25 ± 23.7a x	451.50 ± 38.5a x	0.632	2, 9	0.554
	0.05 VRE-injected	483.25 ± 33.5ab x	474.50 ± 22.8a x	475.00 ± 36.7a x	0.024	2, 9	0.976
	Parasitized	370.25 ± 74.8b x	499.00 ± 17.5a x	475.25 ± 30.6a x	2.059	2, 9	0.184
PL	Untreated	428.00 ± 30.3a x	404.00 ± 9.8ab x	413.00 ± 8.4a x	0.406	2, 9	0.678
	Null-injected	457.00 ± 14.0a x	444.00 ± 23.2abc x	478.00 ± 16.8a x	0.870	2, 9	0.451
	PBS-injected	377.25 ± 27.6a x	396.25 ± 52.7a x	471.00 ± 27.5a x	1.715	2, 9	0.234
	0.005 VRE-injected	454.75 ± 14.1a x	480.75 ± 5.9abc x	459.25 ± 15.5a x	1.220	2, 9	0.340
	0.01 VRE-injected	422.25 ± 17.9a x	440.25 ± 10.5abc xy	529.00 ± 41.2a y	4.611	2, 9	0.042
	0.02 VRE-injected	497.75 ± 10.0ab x	527.25 ± 25.6c x	537.75 ± 38.9a x	0.569	2, 9	0.585
	0.05 VRE-injected	508.50 ± 35.2ab x	517.00 ± 24.8bc x	519.50 ± 36.5a x	0.031	2, 9	0.969
	Parasitized	620.25 ± 75.32b x	490.00 ± 21.11abc x	513.75 ± 34.1a x	1.983	2, 9	0.193
OT	Untreated	11.25 ± 1.7a x	10.00 ± 2.2a x	11.00 ± 2.9a x	0.084	2, 9	0.920
	Null-injected	11.75 ± 4.5a x	10.75 ± 2.4a x	8.75 ± 0.5a x	0.269	2, 9	0.770
	PBS-injected	9.00 ± 1.7a x	20.50 ± 7.3a x	9.00 ± 2.5a x	2.108	2, 9	0.178
	0.005 VRE-injected	10.00 ± 0.7a x	9.50 ± 1.0a x	8.00 ± 0.7a x	1.560	2, 9	0.262
	0.01 VRE-injected	11.50 ± 2.9a x	8.50 ± 1.3a x	9.75 ± 1.0a x	0.607	2, 9	0.566
	0.02 VRE-injected	9.75 ± 3.1a x	8.50 ± 2.6a x	10.75 ± 1.9a x	0.192	2, 9	0.829
	0.05 VRE-injected	8.25 ± 2.4a x	8.50 ± 2.9a x	5.50 ± 1.7a x	0.628	2, 9	0.555
	Parasitized	9.50 ± 1.2a x	11.00 ± 4.4a x	11.00 ± 4.1a x	0.061	2, 9	0.941

[a] Each represents the mean of hemocyte counts from 12 pupae (140 ± 20 mg). GR, granulocytes; PL, plasmatocytes; OT, others.
[b] Numbers in columns (a–c) and rows (x–y) followed by the same letter for each type of hemocytes are not significantly different ($P > 0.05$).

Table 5. ANOVAs of the effects of different treatments, time, and their interactions on the differential hemocyte count of *G. mellonella* pupae

Hemoctye type	Source	df	MS	*F*	*P*	r^2
Granulocytes	Treatment	7	26071.923	7.189	0.000	0.51
	Time	2	5745.510	1.584	0.212	
	Treatment × time	14	5628.891	1.552	0.115	
	Error	72	3626.597			
Plasmatocytes	Treatment	7	27064.161	7.471	0.000	0.52
	Time	2	6478.531	1.788	0.175	
	Treatment × time	14	5721.317	1.579	0.106	
	Error	72	3622.681			
Others	Treatment	7	28.190	0.901	0.510	0.22
	Time	2	22.823	0.730	0.486	
	Treatment × time	14	28.168	0.901	0.562	
	Error	72	31.278			

difference was observed at 24 h ($F = 2.044$; df = 7, 24; $P = 0.091$) (Table 4).

The effect of venom injection and parasitization on DHC of host pupae was treatment but not time dependent for granular cells (treatment: $P = 0.000$; time: $P = 0.212$) and plasmatocytes (treatment: $P = 0.000$; time: $P = 0.175$), and the relationship between treatment and the hemocyte number was not influenced by time for both hemocyte types ($P = 0.115$ and $P = 0.106$, respectively) (Table 5). However, the effects of treatments ($P = 0.510$) and time after treatments ($P = 0.486$) on the number of other hemocyte cell types were not significant. No significant interaction ($P = 0.562$) was observed between treatment and time on the number of other hemocyte cell types (Table 5).

The majority of cells in the total hemocyte population in untreated larvae of *G. mellonella* were granular cells that made up 59.25 ± 1.12, 59.80 ± 3.04, and 57.65 ± 1.52% in the total hemocyte population at three time intervals (4, 8, and 24 h posttreatment). Plasmatocytes were the next most frequent hemocyte type and represented 39.78 ± 1.29, 39.00 ± 3.10, and 41.32 ± 1.53% of the total numbers of hemocytes, respectively (Table 6). Venom injection significantly reduced the number of granulocytes at 4 h ($F = 5.914$; df = 6, 21; $P = 0.001$) in host larvae envenomated by doses >0.02 VRE with respect to controls and lower concentrations of venom. In larvae injected with higher doses of venom (>0.02 VRE), granular cells accounted for <50% in the total hemocyte population whereas this ratio was 59% in untreated individuals at 4 h posttreatment. Significant differences were also observed among treatments in granular cell counts at 8 h ($F = 5.773$; df = 6, 21; $P = 0.001$) and 24 h ($F = 5.443$; df = 6, 21; $P = 0.002$) where the ratio of cells decreased below 50% in the total population at higher doses of venom (Table 6). In contrast, the ratio of plasmatocytes after 4 h significantly increased ($F = 5.880$; df = 6, 21; $P = 0.001$) in larvae envenomated by doses >0.02 VRE compared with 0.02 VRE and the controls. The ratio of plasmatocytes at higher doses of venom (>0.02 VRE) was from 53 to 55%, whereas they exhibited a ratio of 39–43% in untreated and 0.02 VRE injected larvae. A significant variation in the number of plasmato-

Table 6. Differential hemocyte counts (cells/1,000) of *G. mellonella* larvae experimentally envenomated by *P. turionellae*

Hemocyte type	Treatment	Differential hemocyte count (cells/1,000) (mean ± SEM)[a] Time posttreatment (h)[b] 4	8	24	Statistics (ANOVA) *F*	df	*P*
GR	Untreated	592.50 ± 11.2a x	598.00 ± 30.4a x	576.50 ± 15.2ab x	0.293	2, 9	0.753
	Null-injected	595.50 ± 29.8a x	548.00 ± 35.8ab x	617.25 ± 40.6a x	0.986	2, 9	0.410
	PBS-injected	542.50 ± 9.0ab x	556.75 ± 13.0ab x	566.25 ± 15.0abc x	0.903	2, 9	0.439
	0.02 VRE-injected	556.00 ± 48.1ab x	479.25 ± 31.0abc x	523.00 ± 28.7abc x	1.084	2, 9	0.379
	0.05 VRE-injected	457.50 ± 26.1b x	486.25 ± 24.4abc x	547.00 ± 30.2abc x	2.857	2, 9	0.109
	0.1 VRE-injected	455.50 ± 33.6b x	426.75 ± 21.5c x	451.50 ± 15.6c x	0.397	2, 9	0.683
	0.5 VRE-injected	444.75 ± 2.9b x	447.50 ± 19.5bc x	464.75 ± 23.6bc x	0.373	2, 9	0.699
PL	Untreated	397.75 ± 12.9a x	390.00 ± 31.0a x	413.25 ± 15.3ab x	0.308	2, 9	0.742
	Null-injected	392.50 ± 28.5a x	443.50 ± 36.1ab x	374.00 ± 40.6a x	1.032	2, 9	0.395
	PBS-injected	448.00 ± 7.8ab x	433.00 ± 11.9ab x	424.00 ± 15.7abc x	0.980	2, 9	0.412
	0.02 VRE-injected	433.75 ± 50.0ab x	511.75 ± 30.7bc x	467.75 ± 29.1abc x	1.070	2, 9	0.383
	0.05 VRE-injected	535.00 ± 25.7b x	505.50 ± 24.5abc x	444.00 ± 32.0abc x	2.836	2, 9	0.111
	0.1 VRE-injected	534.00 ± 33.9b x	563.50 ± 22.3c x	538.25 ± 16.1c x	0.400	2, 9	0.681
	0.5 VRE-injected	545.00 ± 1.6b x	542.75 ± 17.8bc x	526.25 ± 23.6bc x	0.359	2, 9	0.708
OT	Untreated	9.75 ± 1.8a x	12.00 ± 2.1a x	10.25 ± 2.2a x	0.336	2, 9	0.723
	Null-injected	12.00 ± 3.8a x	8.50 ± 1.3a x	8.75 ± 1.3a x	0.642	2, 9	0.549
	PBS-injected	9.50 ± 1.7a x	10.25 ± 1.7a x	9.75 ± 1.1a x	0.065	2, 9	0.937
	0.02 VRE-injected	10.25 ± 3.6a x	9.00 ± 0.4a x	9.25 ± 0.6a x	0.265	2, 9	0.773
	0.05 VRE-injected	7.50 ± 4.2a x	8.25 ± 1.4a x	9.00 ± 1.8a x	0.267	2, 9	0.771
	0.1 VRE-injected	10.50 ± 2.8a x	9.75 ± 2.1a x	10.25 ± 1.1a x	0.034	2, 9	0.967
	0.5 VRE-injected	10.25 ± 1.4a x	8.00 ± 0.7a x	9.00 ± 0.0a x	0.179	2, 9	0.839

[a] Each represents the mean of hemocyte counts from 12 larvae (260 ± 10 mg). GR, granulocytes; PL, plasmatocytes; OT, others.
[b] Numbers in columns (a–c) and rows (x) followed by the same letter for each type of hemocytes are not significantly different ($P > 0.05$).

Table 7. ANOVAs of the effects of different treatments, time, and their interactions on the differential hemocyte count of *G. mellonella* larvae

Hemocyte type	Source	df	MS	*F*	*P*	r^2
Granulocytes	Treatment	6	42544.968	15.258	0.000	0.63
	Time	2	5930.583	2.127	0.128	
	Treatment × time	12	2646.263	0.949	0.506	
	Error	63	3626.597			
Plasmatocytes	Treatment	6	42853.298	15.072	0.000	0.63
	Time	2	5859.476	2.061	0.136	
	Treatment × time	12	2774.601	0.976	0.481	
	Error	63	2843.238			
Others	Treatment	6	6.631	0.513	0.797	0.10
	Time	2	1.798	0.139	0.871	
	Treatment × time	12	4.298	0.332	0.980	
	Error	63	12.937			

cytes among treatments also appeared at 8 h ($F = 5.902$; df = 6, 21; $P = 0.001$) and 24 h ($F = 5.222$; df = 6, 21; $P = 0.002$) (Table 6). Regardless of time, the number of other hemocyte types did not significantly change among treatments for pupae (4 h: $F = 0.250$; df = 7, 24; $P = 0.967$; 8 h: $F = 1.314$; df = 7, 24; $P = 0.287$; 24 h: $F = 0.718$; df = 7, 24; $P = 0.658$) and larvae (4 h: $F = 0.360$; df = 6, 21; $P = 0.896$; 8 h: $F = 0.868$; df = 6, 21; $P = 0.534$; and 24 h: $F = 0.218$; df = 6, 21; $P = 0.967$).

The effect of venom injection on DHC of host larvae was treatment but not time dependent for granular cells (treatment: $P = 0.000$; time: $P = 0.128$) and plasmatocytes (treatment: $P = 0.000$; time: $P = 0.136$), and the relationship between treatment and the hemocyte number was not influenced by time for both hemocyte types ($P = 0.506$ and $P = 0.481$, respectively) (Table 7). However, the effects of treatments ($P = 0.797$) and time passed after treatments ($P = 0.871$) on the number of others were not significant. No significant interaction ($P = 0.980$) was observed between treatment and time on the number of others (Table 7).

Hemocyte and Cell Assays. Hemocytes were collected from last instars of G. *mellonella* and seeded in TC-100 media containing 10% FBS in 96-well plates. By 1 h, the majority of hemocytes present were granular cells that were attached to the culture plates but not spread. Plasmatocytes represented the second most abundant cell type, and most of these cells were beginning to spread over the plate surface. Upon addition of saline, granular cells and plasmatocytes displayed spreading behavior within 1–2 h of treatment (Fig. 2), and by 24 h, the majority of both cell types were tightly adhered and spread thinly across the plate surface. By contrast, addition of an LC_{99} dose of venom (0.001 VRE/μl) induced some vacuole formation in both plasmatocytes and granular cells within 15 min of treatment. At 45 min posttreatment, venom treated granular cells became much more dense or granular throughout the cytoplasm, and extensive vacuole formation was evident (Fig. 2). Approximately 40% ($n = 9340$ cells counted) of the granular cells were dead at this time point, and membrane blebs surrounded the external periphery of these cells. Plasmatocytes also displayed vacuole formation but nearly all remained attached to cell culture plates and none seemed dead. By three and 6 h posttreatments, almost no changes in cellular effects were observed. In fact, all of the granular cells did not die until 24 h posttreatment. None of the plasmatocytes died during the test period.

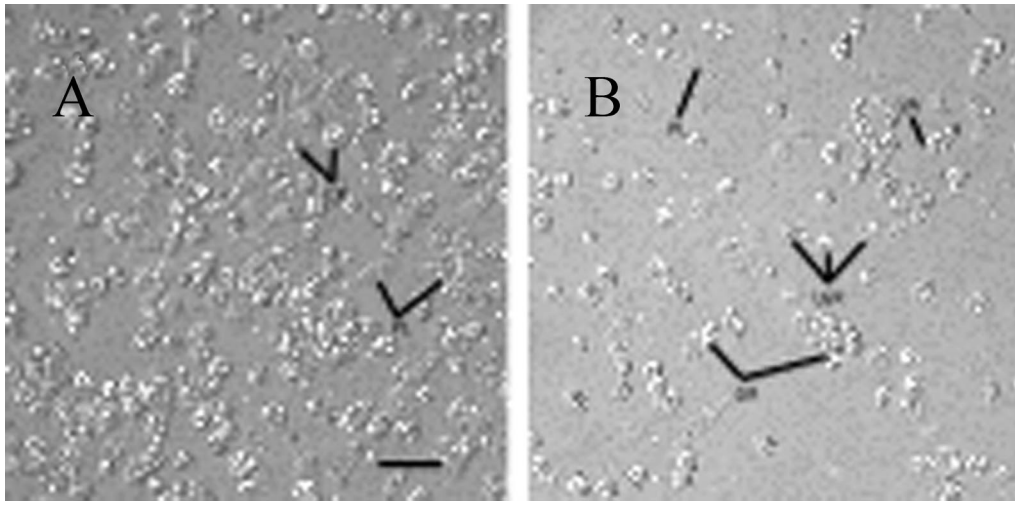

Fig. 2. Phase-contrast micrographs of adhesive hemocytes from last instars of *G. mellonella* cultured in vitro with isolated crude venom (0.001 VRE/μl) from *P. turionellae*. Hemocytes were incubated in vitro at 27°C for 1 h in TC-100 medium with 10% FBS before the addition of phosphate isolation buffer (A) or venom (B). Photomicrographs were taken at 45 min posttreatment. GR, granular cells; PL, plasmatocytes; LGR, lysed granular cells; VA, vacuoles. Bar = 20 μm.

Discussion

Insect immunity is characterized by innate responses involving both cellular and humoral mechanisms (Carton and Nappi 2001, Schmidt et al. 2001). Most studies associated with host-parasite relationships, especially those of endoparastic wasps, have been focused on the functionality of encapsulation processes (Schmidt et al. 2001). Inhibition of host immune responses is critical for endoparasitic species to ensure that eggs and larvae are not recognized and eliminated by the cellular-arm of the innate defenses. Protection for wasp progeny generally is achieved through disabling host hemocytes. In this study, we show that parasitism by *P. turionellae* elicits a sharp drop in the number of circulating hemocytes in pupae, and to a lesser extent larvae, of a natural host *G. mellonella.* Artificial injection of isolated venom into hosts achieved nearly identical morphological, behavioral, and viability changes as parasitism, implicating venom as the agent that targets host hemocytes.

Parasitism and experimental envenomation of *G. mellonella* by *P. turionellae* resulted in markedly different effects on the number of hemocytes circulating in hemolymph depending on the host developmental stages. Depending on venom dose or length of time after exposure to venom, total hemocyte counts of parasitized or envenomated pupae were lower than controls and parasitized or venom injected larvae. The drop in cell numbers in venom-treated and parasitized hosts seemed to be due to hemocyte death. *P. turionellae* displays a broad host range (Kansu and Uğur 1984) and can successfully oviposit in multiple life stages of the same hosts. Similarly, isolated venom has been shown to be toxic to a broad range of insects, including multiple developmental stages and cell types (Ergin et al. 2006). Despite the broad action of activity, venom does show some specificity in terms of host developmental stage based on our observations of hemocyte viability. In fact, the higher susceptibility of pupal hemocytes to parasitism and venom injection is consistent with the oviposition preference of adult females, which select pupae over larva when given a choice. Avoidance of nonpermissive or less submissive hosts is expected for endoparasitic wasps that depend on immunosuppression as part of host conditioning (Carton and Nappi 2001).

In several lepidopteran hosts, successful parasitization by parasitic wasps leads to a reduction in the total number of hemocytes in circulation (Mochiah et al. 2003, İbrahim and Kim 2006). Decreases in hemocyte numbers and increases in hemocyte damage also occurred in experimentally envenomated insects (Richards and Edwards 1999, Zhang et al. 2005a, Yu et al. 2007). In contrast, Zhang et al. (2005b) reported that parasitism by *P. puparum* resulted in a noticeable increase in total hemocyte numbers of its two hosts for a defined period. The same trend was also observed before by Prévost and Eslin (1998) who reported an increase of hemocyte counts in larvae from six *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) subgroup species after parasitism by *Asobara tabida* Nees (Hymenoptera: Braconidae). Studies with endoparasitic wasps indicate that in some cases hemocyte number, morphology and viability may be affected by PDVs (Beckage 1998). Microscopic analysis of tissues associated with the female reproductive tract of *P. turionellae* has provided no evidence of PDVs in this wasp; thus, it seems that some other factor (presumably venom alone) is responsible for the changes in host hemocytes.

Effects of parasitization on host hemocyte immune systems have been documented for a number of larval parasitoids (Stettler et al. 1998). In the *Campoletis sonorensis* (Cameron)/*Heliothis virescens* (F.) system, the number of granular cells increased whereas plasmatocytes were reduced after parasitization but not affected by venom injection (Davies et al. 1987). In *Microplitis demolitor* (Wilkinson)/*Pseudoplusia includens* (Walker) (Strand and Noda 1991) and *Chelonus inanitus* L./*Spodoptera littoralis* Boisduval (Stettler et al. 1998), no obvious effect was seen after parasitization. In contrast, the effect of parasitism on DHC has been investigated only in pupal hosts; Cai et al. (2004) observed a significant decrease in the percentage of plasmatocytes occurred after parasitization of *Pieris rapae* by the pupal endoparasitoid *P. puparum*, while granular cells increased significantly. Venom from *P. puparum* displayed significantly higher activities toward plasmatocytes and granular cells from both larvae and pupae of two natural hosts, *P. rapae* and *Papilio xuthus* L. (Lepidoptera: Papilionidae), and lower activity toward those from *Spodoptera litura* F. (Lepidoptera: Noctuidae), *Musca domestica* L. (Diptera: Muscidae), and *Sarcophaga peregrina* (Robineau-Desvoidy) (Diptera: Sarcophagidae) (Zhang et al. 2005a). However, no effect was found toward any type of hemocytes from the five insect species tested (Zhang et al. 2005a). In the current study, both venom from and parasitization by *P. turionellae* showed potent effects on *G. mellonella* pupal and larval hemocytes, arguing that the wasp's venom has a broader spectrum of activity than parasitoids that target a single host developmental stage.

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