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Effects of the endoparasitoid *Cotesia chilonis* (Hymenoptera: Braconidae) parasitism, venom, and calyx fluid on cellular and humoral immunity of its host *Chilo suppressalis* (Lepidoptera: Crambidae) larvae

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ABSTRACT

The larval endoparasitoid *Cotesia chilonis* injects venom and bracoviruses into its host *Chilo suppressalis* during oviposition. Here we study the effects of the polydnavirus (PDV)-carrying endoparasitoid *C. chilonis* (Hymenoptera: Braconidae) parasitism, venom and calyx fluid on host cellular and humoral immunity, specifically hemocyte composition, cellular spreading, encapsulation and melanization. Total hemocyte counts (THCs) were higher in parasitized larvae than in unparasitized larvae in the late stages following parasitization. While both plasmatocyte and granulocyte fractions and hemocyte mortality did not differ between parasitized and unparasitized hosts, *in vitro* spreading behavior of hemocytes was inhibited significantly by parasitism throughout the course of parasitoid development. *C. chilonis* parasitism suppressed the encapsulation response and melanization in the early stages. Venom alone did not alter cellular immune responses, including effects on THCs, mortality, hemocyte composition, cell spreading and encapsulation, but venom did inhibit humoral immunity by reducing melanization within 6 h after injection. In contrast to venom, calyx fluid had a significant effect on cell spreading, encapsulation and melanization from 6 h after injection. Dose–response injection studies indicated the effects of venom and calyx fluid synergized, showing a stronger and more persistent reduction in immune system responses than the effect of either injected alone.

1. Introduction

To survive and develop in the hemocoel of insect hosts successfully, endoparasitoids have evolved spectacular mechanisms to avoid or inhibit both the cellular and humoral immune responses of their hosts (Asgari, 2006; Burke and Strand, 2014; Hoffmann et al., 1999; Kanost and Gorman, 2008; Schmidt, 2008; Strand, 2008). Endoparasitoids display diverse strategies in different parasitoid-host systems (Dorémus et al., 2013a) to avoid being recognized as foreign objects by host immune systems. These include a "passive" strategy that is more accurately termed "local active regulation" whereby parasitoid eggs are often covered by some protective components (Asgari et al., 1998; Furihata et al., 2014; Hu et al., 2014; Dorémus et al., 2013a).

As well, endoparasitoids also have evolved "active strategies" whereby the injection of maternal factors with eggs into the cavity of the host can alter the host immune physiology. These factors include venoms (Asgari and Rivers, 2011), polydnaviruses (PDVs) (Gundersen-Rindal et al., 2013), virus-like particles (VLPs) (Reineke et al., 2006) and ovarian proteins (Webb and Luckhart, 1996), and the effect of each depends on the parasitoid species (Asgari, 2006; Beckage, 2008; Schmidt, 2008). Among these maternal parasitoid factors, PDVs are the most studied. PDVs persist as stably integrated proviruses in the genome of associated wasps and replicate only in the calyx cells of female ovaries. From the ovary, virions are then transferred into a host during oviposition and rapidly infect host cells. As a result of the expression of the viral products, a myriad of physiological changes can be observed, notably the inhibition of the immunity of the host. PDVs never replicate in the host, because the packaged DNA lacks virus-related genes required for replication (Gundersen-Rindal et al., 2013; Strand, 2010).

Together with PDVs, a fluid from the venom gland is injected into the host along with the eggs. Venom can induce complex physiological alterations (Asgari and Rivers, 2011; Pennacchio and Strand, 2006). For endoparasitoids lacking PDVs, venom is

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the main source to regulate the host immunity, including both cytotoxic and cytolytic effects (Er et al., 2010, 2011; Parkinson et al., 2002a,b, 2004), and alterations in host cellular and humoral immunity (Cai et al., 2004; Colinet et al., 2009; Fang et al., 2011a,b; Labrosse et al., 2005; Mabiala-Moundoungou et al., 2010). However, in some PDV-carrying endoparasitoids, especially in ichneumonids, venom is not essential (Beckage et al., 1990; Davies et al., 1987; Dorémus et al., 2013b; Stoltz and Guzo, 1986; Strand and Noda, 1991), while in some braconids the venom is necessary to protect the eggs from encapsulation (Kitano, 1986; Wago and Tanaka, 1989), reduce the proteolysis of hemolymph prophenoloxidase (Asgari et al., 2003a,b) and promote the persistence and expression of PDVs (Stoltz et al., 1988; Zhang et al., 2004). Up to now, there have been few rigorous analyses of the continuous effects of parasitism, in particular the effects of different maternal factors, on both host cellular and humoral immunity; moreover, the long-term interaction of venoms and PDVs has been subject only to limited investigations.

Cotesia chilonis (Hymenoptera: Braconidae) is an obligate larval endoparasitoid that effectively regulates the density of *Chilo suppressalis* (Lepidoptera: Crambidae), one of the economically most important rice pests in China and other Asian countries. This parasitoid is mainly present in China, Japan, Indonesia and North Korea and its natural parasitism rates range from 10% to 30% and may be as high as 90%, for example, in Jiangsu province, China (Lou et al., 2014; Qi et al., 2014). This braconid has also been imported into several African countries as a means to control lepidopteran stem borers (Hailemichael et al., 2008). Our previous studies indicated that *C. chilonis* injected bracovirus and venom into hosts during oviposition (Li et al., 2011) to influence host expression of fatbody and hemocyte genes (Qi et al., 2014; Wu et al., 2013). However, little is known about the influence of *C. chilonis* parasitization and parasitoid-associated factors on the host *C. suppressalis* immune system. In this study, we described the immune reactions of *C. suppressalis* parasitized by *C. chilonis* and injected with factors derived from the adult female wasps. Long-term dose–response studies were performed to investigate the interaction between venom and calyx fluid and our results contribute to a better understanding of the complementarity between injected factors that regulate the interaction between parasitoid wasps and their hosts.

2. Materials and methods

2.1. Insect collection, rearing and parasitization

Host *C. suppressalis* moths were collected primarily from paddy fields in the experimental farmland of China National Rice Research Institute in Fuyang (30.07°N, 119.95°E), Hangzhou, China. The laboratory colony was reared on artificial diet (Han et al., 2012). The endoparasitoid *C. chilonis* colony was reared on host *C. suppressalis* larvae. Both species were maintained under the conditions of 28 ± 1 °C, 70–80% relative humidity, and a 16 h light-8 h dark photoperiod. For parasitism experiments, 4th instar host larvae aged 1 day old were exposed to one-day-old mated female wasps, which had no previous contact with hosts. To avoid superparasitism, one host larva was held together with just one mated female wasp in a petri dish, and the parasitoid was removed immediately after a single oviposition was observed. The parasitized larvae were then cultured under the conditions mentioned above. The *C. chilonis* larvae emerged from the *C. suppressalis* larvae 9 days after parasitism under the conditions mentioned above, and host could retain viability for 4–5 days with a weak vitality. All solutions, materials used in this study were sterilized. The 4th instar larvae aged 1 day old were used for the experiments associated with parasitism or injection with different wasp maternal factors.

2.2. Injections of venom and calyx fluid

Venom and calyx fluid collection procedures were described by Strand and Noda (1991). Briefly, prepared female wasps were individually swabbed with 75% ethanol (v/v), dried and then the reproductive tracts of wasps were excised under sterile Pringle's phosphate-buffered saline (PBS) with an aid of a Leica MZ 16A stereomicroscope (Leica, Germany). Venom apparatuses and calyces were removed and placed separately into PBS drops. The venom reservoirs and calyces were torn open with forceps, respectively, and then venom apparatuses, calyx tissues and eggs were removed. PBS drops containing venom or calyx fluid were transferred into 1.5 ml eppendorf tubes and then centrifuged respectively at 8000*g* or 200*g* for 10 min at 4 °C. After centrifugation, we obtained supernatants containing either venom or calyx fluid. We mixed venom and calyx fluid supernatants to obtain a sample with both fluids and all supernatants (venom, calyx fluid and venom plus calyx fluid) were diluted with PBS into appropriate concentrations required for injections. The venom, calyx fluid and mixture of venom and calyx fluid injected into a host were 0.01, 0.15, and 0.75 wasp equivalents. One wasp equivalent was defined as parasitism factors injected by only one wasp. Host larvae were anaesthetized with carbon dioxide and injected through a proleg with a glass micropipette mounted on a micromanipulator.

2.3. Hemocyte counts, mortality and cell spreading ability

Total hemocyte count (THC), differential hemocyte count (DHC), mortality and cell spreading ability were measured from individual *C. suppressalis* larva parasitized/injected with 1 μl, 0.01, 0.15, 0.75 equivalents of venom, calyx fluid and venom plus calyx fluid at different sampling periods, respectively. Unparasitized larvae injected with 1 μl of PBS were used as controls. Larvae were anaesthetized with carbon dioxide, surface sterilized with 75% ethanol (v/v), dried and then bled onto Parafilm® by cutting away a proleg. 100 μl of 10× diluted hemolymph from five larvae with the same treatment was collected into a sterilized glass tube as a pool. There was a total of five pools for one treatment.

THC was performed by transferring 1 μl of 10× hemolymph from one pool to Neubauer hemocytometer and by counting under the phase contrast microscope (Nikon eclipse TS-100, Japan). DHC observation was performed by incubating 1 μl of 10× diluted hemolymph from one pool into a well of 96-well plates (Corning Incorporated costar® 3599, Corning, USA) containing 150 μl TC-100 insect medium (PAN-Biotech, Germany) with 10% fetal bovine serum for 1 h and calculating the fraction of plasmatocytes and granulocytes with the same model of microscope. Approximately 150 cells were identified from three randomly selected fields using criteria specified by Strand (2008). While granulocytes were morphologically distinguished based on the appearance of both cytoplasmic granules and spreading behavior, plasmatocytes were usually larger than granulocytes and spread asymmetrically with lamellipodia. Cellular mortality was confirmed using the CellTox™ Green Dye (Promega, USA), which exhibits enhanced fluorescence when stably bound to the DNA of cells that have lost membrane integrity as a result of cell death in contrast to viable cells, which produce no appreciable increase in fluorescence. In this assay, 1 μl of 10× diluted hemolymph from one pool was added to a well of 96-well plates containing 150 μl TC-100 insect medium with 10% fetal bovine serum for 15 min and shielded from light. Then the ratios of green fluorescent hemocytes were calculated from five randomly selected fields of the view under the wavelength 450–490 nm using a Nikon eclipse TS-100 (Nikon Intensilight C-HGFI) to detect fluorescence.

Spreading behavior was measured by incubating 1 μl of 10× diluted hemolymph from one pool into a well of 96-well