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Ultrastructural and functional characterization of circulating hemocytes from *Galleria mellonella* larva: Cell types and their role in the innate immunity

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ABSTRACT

Galleria mellonella larvae have been widely used as a model to study the virulence of various human pathogens. Hemocytes play important roles in the innate immune response of *G. mellonella*. In this study, the hemocytes of *G. mellonella* larvae were analyzed by transmission electron microscope, light microscope, and cytochemistry. The cytological and morphological analyses revealed four types of hemocytes; Plasmatocytes, granular cells, spherule cells and oenocytoids. Differential hemocyte counts showed that under our conditions plasmatocytes and granular cells were the most abundant circulating cell types in the hemolymph. We also investigated the role of different types of hemocytes in the cellular and humoral immune defenses. The in-vivo experiment showed that plasmatocytes, granular cells and oenocytoids phagocytized FITC-labelled *Escherichia coli* bacteria in larvae of *G. mellonella*, whereas the granular cells exhibited the strongest phagocytic ability against these microbial cells. After incubation with L-DOPA, plasmatocytes, granular cells and oenocytoids are stained brown, indicating the presence of phenoloxidase activity. These results shed new light on our understanding of the immune function of *G. mellonella* hemocytes.

1. Introduction

Insects lack an acquired immune system but they nevertheless possess strong complex innate immune systems including cellular and humoral defenses. Cellular defenses refer to phagocytosis, nodulation, and encapsulation which are mediated by hemocytes (Lavine and Strand, 2002; Negri et al., 2014). Hemocytes recognize foreign targets either by direct interaction of their pathogen recognition proteins (PRRs) with the pathogen-associated molecular patterns (PAMPs) from the invading species, or, indirectly, by recognition of humoral immune effectors (Ohta et al., 2006). Based on morphological, cytochemical, and functional characteristics, several types of hemocytes have been identified in insects (Giulianini et al., 2003; Huang et al., 2010; Soares et al., 2013). The most common types of hemocytes are prohemocytes, plasmatocytes, granular cells, spherule cells, and oenocytoids (Ribeiro and Brehelin, 2006; Strand, 2008). However, the immune functions of hemocytes vary between insect species, infectious challenges, and according to developmental stage (Negri et al., 2014).

Because it is widely considered ethically preferable to use invertebrates rather than mammals in studies of infection, insects have been widely used as models for preclinical toxicological studies (Giannouli et al., 2014; Kanost et al., 2004; Loh et al., 2013). *Galleria mellonella* is a good experimental animal for the following reasons: the size of the larvae is suitable for processing; they can be reared at various temperatures (20 °C to 37 °C), allowing experiments that mimic a mammalian environment; and *G. mellonella* larvae are easy to culture and are inexpensive to purchase (Kanost et al., 2004; Ramarao et al., 2012). *G. mellonella* larvae have been used as a model for the identification and characterization of microbial virulence factors involved in mammalian infections (Desbois and Coote, 2012). Hematology is an integral part of immunology studies on animals, and a good knowledge of hemocytes, therefore, is required before insects can reach a level of importance similar to that of vertebrate models in terms of infection and immunity (Berger and Slavickova, 2008; Kanost et al., 2004).

Here, we deepen the knowledge of *G. mellonella* hemocytes by providing extensive morphological and functional characteriza-

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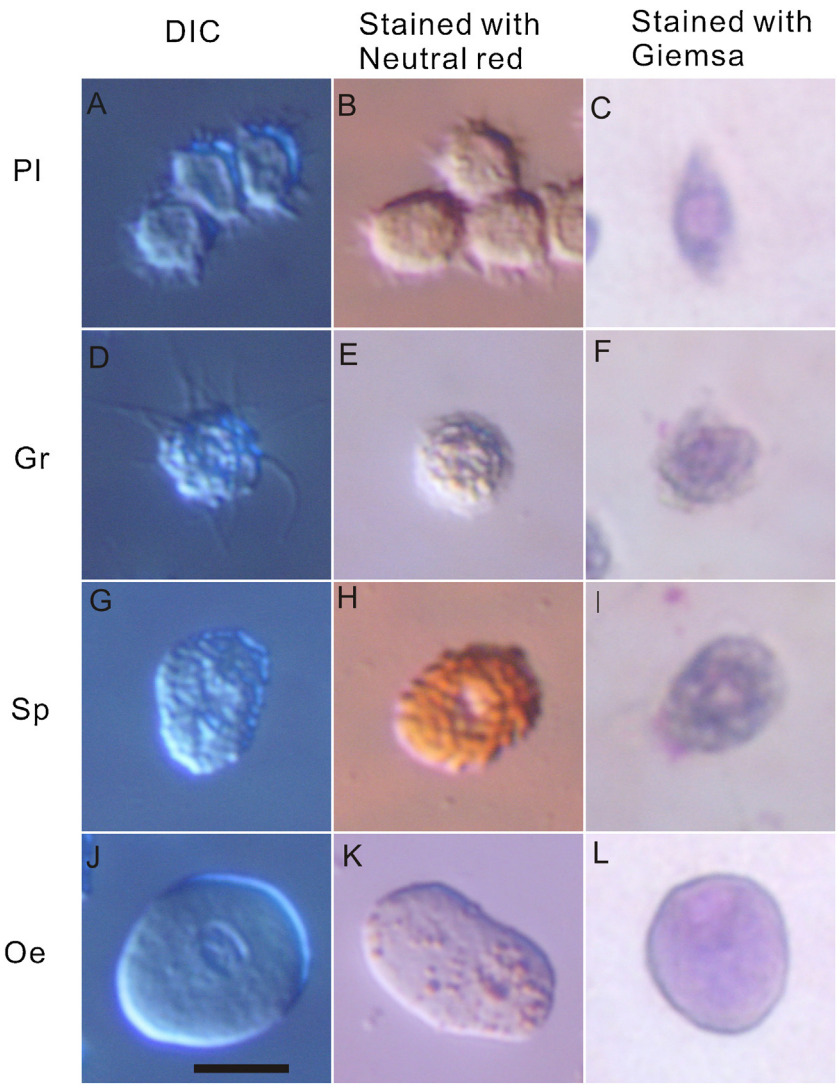

Fig. 1. Light micrographs of hemocytes of larval *Galleria mellonella* stained with Giemsa or neutral red. PL: plasmatocyte; Gr: granular cell; Sp: spherule cell; Oe: oenocytoid. Bar = 10 μm.

tion, based on electron and light microscope. We provided evidence showing that the oenocytoids are involved in phagocytosis and deciphered the role of different hemocytes in *G. mellonella* innate immunity. We also examined the proportion of different hemocyte types in the hemolymph at different developmental stages of *G. mellonella*.

2. Materials and methods

2.1. Insects

The larvae of the greater wax moth *G. mellonella L.*, (Lepidoptera: Pyralidae) were reared on an artificial diet as described previously (Wu and Yi, 2015). The instars of each larvae were determined by measuring the head capsule width according to previous studies (Garedew et al., 2004; Irigaray et al., 2006).

2.2. Characterization of hemocytes

For light microscope (LM) analysis, the last instars larvae were selected and placed on ice (1–2 min) for immobilization. The hemolymph of ten larvae was obtained by pricking the larvae with an insect needle and bled directly on to a glass slide and allowed to dry in natural air conditions for 10 min. Cells were then fixed in methanol for 10 min. After natural air-drying of the fixative, hemocytes were stained with Giemsa or neutral red for 10 min and slides were rapidly washed with phosphate buffered saline (PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 1000 ml distilled water, pH 7.2). The cells were identified by light microscope or phase-contrast microscope at 40× magnification and images were acquired with a photo camera.

For transmission electron microscope (TEM) analysis, hemolymph of at least 100 last instars larvae was collected on ice. The hemolymph obtained was pooled and centrifuged at 500*g* for 5 min. The pellet was resuspended and fixed in 4% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) overnight. The samples were washed in 5% sucrose solution in 0.2 M cacodylate buffer (pH 7.2) and post-fixed with osmium tetroxide in cacodylate buffer for 1 h. After dehydration in graded acetone series, the cells were embedded in Epon 812. Sections were cut on an ultramicrotome (Pabisch Top Ultra 150) and collected on 200 mesh nickel grids. Following staining with uranyl acetate and lead citrate for 5 min each, sections were observed with a JEOL JEM-1400 transmission electron microscope.