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Orijinal araştırma (Original article)

A new approach for classification of major larval hemocytes (prohemocytes, plasmatocytes and granulocytes) in the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae*)* by acridine orange staining

Büyük bal mumu güvesi *Galleria mellonella* L. (Lepidoptera: Pyralidae)'nın başlıca larval hemositlerinin (prohemositler, plazmatositler ve granülositler) akridin oranj boyama ile sınıflandırılması için yeni bir yaklaşım

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Summary

Larval hemocytes of the greater wax moth, *Galleria mellonella* L. 1758 (Lepidoptera: Pyralidae), were classified by fluorescence microscopy staining with acridine orange. Based on their fluorescence characteristics, plasmatocytes and prohemocytes could be easily distinguished from granulocytes because they appear faint green and do not contain any fluorescent granules in their cytoplasm. Nuclei of granulocytes emit bright green fluorescence when observed under fluorescence microscope. Prohemocytes are round and their nuclei are bright. Plasmatocytes are irregularly shaped. Therefore, all three types of the wax moth's major hemocytes that are difficult to identify by bright field or phase contrast microscopy can be easily classified by fluorescence microscopy with staining acridine orange. Furthermore, this technique will assist further studies to understand how hemocyte differentiation and regeneration proceeds in larval hematopoietic organs and during hemocyte transformation. The fluorescent method for hemocyte classification is more precise than the common method of hemocyte identification using bright field or phase contrast microscopy.

Key words: *Galleria mellonella,* hemocyte, acridine orange, fluorescence microscopy

Özet

Büyük mum güvesi, *Galleria mellonella* L. 1758 (Lepidoptera: Pyralidae)'nın larval hemositleri acridine orange ile boyanarak floresans mikroskopta sınıflandırılmıştır. Hemositlerin floresan özelliklerine göre, plasmatositler ve prohemositler, granulositlerden kolaylıkla ayırt edilebilir. Çünkü plasmatositler ve prohemositler soluk yeşil görünürler ve sitoplazmalarında herhangi bir floresan granülleri içermezler. Granulositler floresans mikroskop ile incelendiğinde nukleusları parlak yeşil ışıma yaparlar. Prohemositler yuvarlaktır ve nukleusları parlaktır. Plasmatositlerin düzensiz şekilleri vardır. Büyük mum güvesinin en önemli üç tip hemositlerin parlak alan ve faz kontrast mikroskop ile tanımlanmaları zor olduğundan dolayı, hemositler acridine orange boyası ile floresans mikroskop altında sınıflandırılabilir. Ayrıca, bu teknik hemositlerin dönüşümü sırasında ve hematopoietik organda hemositlerin nasıl farklılaştığının anlaşılması için yapılan ileri çalışmalara destek sağlayacaktır. Hemositlerin sınıflandırılmasında kullanılan floresan tekniği, genel bir yöntem olan parlak alan ve faz kontrast mikroskop ile yapılan hemosit belirlenmesine göre daha hassas bir yöntemdir.

Anahtar sözcükler: *Galleria mellonella,* hemosit, akridin oranj, floresan mikroskobu

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Introduction

Research on insect hemocytes has received much attention because they are the cells that mediate insect cellular immunity (Huang et al., 2010) and also provide an excellent model system for the study of cell development, differentiation and communication (Ling et al., 2003). During the cellular immune response, hemocytes are able to distinguish self from non-self, reacting pathogens, parasites or eggs by phagocytosis (engulfment of organisms by single hemocytes), multicellular encapsulation and nodule formation (Ratcliffe et al., 1985; Lackie, 1988; Willott et al., 1994; Yokoo et al., 1995; Trenczek & Kanost, 1997; Jarosz, 1998; Vilkinskas & Götz, 1999; Karaçalı et al; 2000; Ling & Yu, 2006; Strand, 2008). The immune response of caterpillars, including. *Galleria mellonella, Manduca sexta, Bombyx mori*, depends on the activities of two main hemocyte populations, granulocytes and plasmatocytes that recognize pathogens and parasites (Nardi et al., 2003). They are the effector cells of the immune systems (Tojo et al., 2000), comprising approximately 90% of all hemocytes (Nardi, 2004; Levin et al; 2005; İzzetoğlu & Karaçalı, 2010). In Lepidoptera, plasmatocytes are the predominant cells with phagocytic activity. Although granulocytes represent the most abundant hemocytes, they possess little phagocytic activity (Lackie, 1988; Pech & Strand, 1996; Karaçalı et al.; 2000; Tojo et al., 2000; Strand, 2008).

With renewed research on larval hemocytes, accurate classification of these cells is an important prerequisite for these studies. The terminology applied to hemocytes of different species has often complicated comparisons of hemocyte classes in different insect orders. (Nardi, 2004; Huang et al., 2010). Although larval hemocytes of Lepidoptera are typically identified by field or phase microscopy, this conventional method of hemocyte classification has been the source of frequent controversy (Ling et al.; 2003). For other insect orders, the hemocyte terminology is likewise based on morphological features, but these features often differ from order to order. For example, in insects, there were until recently over 70 different names in use for just 6-9 hemocyte types (Ratcliffe et al., 1985). There is clearly also a need to develop a more uniform terminology for naming insect hemocytes in different species (Strand, 2008).

Insect hemocytes have historically been identified and classified using morphological, histochemical and functional characteristics (Strand, 2008). The recent use of monoclonal antibodies (mAbs) to identify insect hemocytes is an exciting development because of the promise of high specificity (Chain et al.,1992; Mullett et al., 1993; Willott et al., 1994; Gardiner & Strand, 1999). However, mAbs specific enough for identification of each hemocyte type have not been isolated yet. A disadvantage of the use of mAbs or enzyme activity in the identification of larval hemocytes is the unavoidable repeated washing steps which may result in the loss of hemocytes from glass coverslips or slides. Therefore, the need to develop an exact and easy way to classify insect hemocytes still exists. Ling et al. (2003) first showed that a fluorescent cationic dye, acridine orange, that is specific for nucleic acids and useful for cell cycle determination could also be used for classifying types of hemocytes. Moreover, Huang et al. (2010) described the hemocyte classes of *Plutella xylostella* (Lepidoptera) according to their affinity for this dye. Here we also show that the plasmatocytes, prohemocytes and granulocytes of *Galleria mellonella* can be classified by staining with acridine orange.

Materials and Methods

Experimental animals

The greater wax moth larvae of *Galleria mellonella* Linnaeus 1758 (Lepidoptera: Pyralidae) were used in this study. Larvae were reared on an artificial diet (Oberlander et al., 2000) at 30 ^{o}C in darkness. Natural relative humidity was maintained within large Petri dishes. Hemocytes (insect blood cells) obtained from last instar larvae were used for the experiments. Hemocytes were classified into three morphotypes using the criteria of Lackie (1988).

Cell culture of hemocytes

The cell culture medium was composed of Grace's Insect Medium (Sigma, G8142) with 10% fetal calf serum (Sigma, F3018) and antibiotic-Ampicillin (AppliChem-A0839,0025). The cultures were maintained at 26 ^{o}C with natural humidity. Hemocytes adhered to sterile cover glasses (22 mm^2) that

were placed on the bottom of small Falcon culture dishes (35x10 mm) containing Grace's insect medium. Circulating hemocytes from last instar larvae were added to disposable culture dishes. As soon as the drop of larval hemolymph touched the medium, it was swirled, and cells were allowed to settle and adhere to the glass coverslip for 1 h.

Use of acridine orange to stain hemocytes for classification by fluorescence microscopy

Hemocytes were cultered and then stained as described by Ling et al. (2005), with the modifications listed below. To maintain the physiological environment of hemocytes, larval insect saline (130 mM NaCl, 5.0 mM KCl, and 1.0 mM $CaCl_2$; pH 7.0) was used to dissolve acridine orange. After the culture period, the medium and unattached hemocytes were removed from the culture dishes. To observe hemocytes types, 100 µl acridine orange (10 µg/ml) was dropped onto a slide,and covered with a coverslip on which adhered living hemocytes. Hemocytes stained with acridine orange were viewed with the appropriate fluorescence blue filters using a differential interference fluorescence microscope (Leica DM 4000B with DP71 video camera System).

Results and Discussion

In Lepidoptera, classification of hemocytes has historically been based on their morphology at the light microscope level (Ratcliffe et al., 1985). Although more precise characterization of each type of hemocyte at the electron microscope level was also done (Akai & Sato 1973), classification of hemocytes still depends upon light microscope examination. A comprehensive classification of hemocytes types is difficult because of their different appearances under different culture conditions and after a variety of processing techniques (Huang et al., 2010). In the wax moth, *Galleria mellonella*, Shrivastava and Richards (1965) demonstrated the presence of at least three types of hemocytes; prohemocyte, granulocyte (granular cells) and plasmatocyte. Identification of each type by light microscopy has often been perplexing, especially for granulocytes which are difficult to distinguish from prohemocytes, even for experienced researchers (Ling et al., 2003; Ling et al., 2005). When observed under the light microscope, the differences between some granulocytes and prohemocytes of *G. mellonella* is not always obvious, especially when they are comparable in size (Fig. 1a).

Ling et al. (2003) found that acridine orange could be used to classify the circulating hemocytes of the silkworm, *Bombyx mori* (Lepidoptera). This method is extremely useful for the discrimination of specific cells that are difficult to identify by ordinary light microscopy. When stained with acridine orange (AO), granulocytes and spherulocytes are easily distinguished from prohemocytes and plasmatocytes based on the presence of bright green fluorescent granules in cytoplasm (Ling et al., 2003; Ling et al., 2005). Granulocytes stained with AO contain many bright green granules, and their nuclei emit bright green fluorescence when observed under a fluorescence microscope. In granulocytes, these granules are irregularly shaped and vary in size. In *Galleria mellonella*, many granulocytes are not easily distinguished from prohemocytes by light microscopy (Fig. 1a). However, using fluorescence microscopy, granulocytes can be easily distinguished from the prohemocytes and plasmatocytes; neither of the latter two hemocyte types contain fluorescent granules but both appear pale green (Fig. 1b, c). Plasmatocytes are very variable in shape and their plasma membranes exhibit irregular processes: filopodia and pseudopodia (Fig. 1c, d). Prohemocytes are round shaped. In the silkworm, *B. mori*, the nuclei of prohemocytes and plasmatocytes appear dark (Ling et al., 2003). However, the nuclei of prohemocytes and plasmatocytes in *G. mellonella* emit bright green fluorescence (Fig. 1d).

Fluorescence microscopy is particularly well suited to perform structure-function analysis of living cells using vital staining with fluorescent probes (Canete et al., 2001). Among the commonly used fluorescent probes, AO is one of the most widely used dyes for analysis of cell viability and selective visualization of organelles and dead cells (Canete et al., 2001; Foglieni et al., 2001). In addition to visualization of lysosomes, AO has been used for vital staining of blood parasites and for discriminating between living and dead cells of bacteria, yeasts and mammals. (Canete et al., 2001). AO can readily enter living cells and shows preference for normal AT-rich regions of nuclei. Healthy cells have green nuclei and their cytoplasms stain pale green with AO (Foglieni et al., 2001). Hemocytes positive for AO

are dead or dying cells. Presumably granulocytes whose cytoplasms stain intensely with AO are also apoptotic. The AO-positive granules found in these cells of *B. mori* are apoptotic bodies (Ling et al., 2005). Since granulocytes of *G. mellonella* are phagocytic cells as well (Karaçalı et al., 2000), granulocyte granules that can be more positively stained are probably phagocytosed apoptotic bodies.

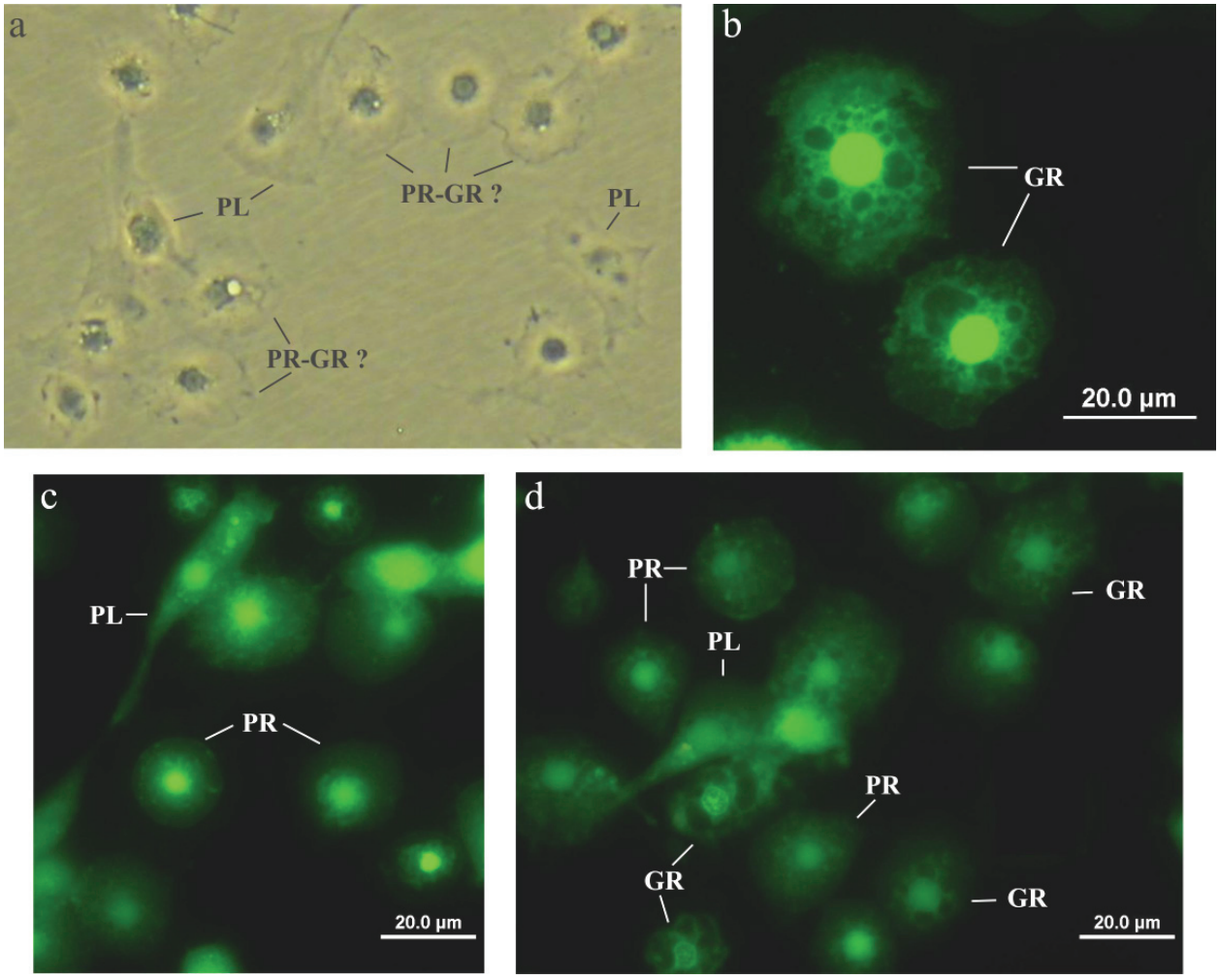

Figure 1. Morphology and comparison of the hemocytes of *Galleria mellonella* larvae with inverted and fluorescence microscopy. (a) When observed under the light microscope, the difference between some granulocytes and prohemocytes is not always obvious, especially when they are comparable in size. (b, c, d) Morphology of prohemocytes, plasmatocytes and granulocytes revealed by staining with acridine orange under fluorescence microscopy. Following staining with acridine orange, plasmatocytes and prohemocytes can be easily distinguished from granulocytes because they generally appear faint green and do not contain fluorescent granules in their cytoplasms. In granulocytes, these granules are irregularly shaped and vary in size. (PL: plasmatocye, PR: prohemocytes, GR: granulocyte, No scale bar is given for Fig. 1a).

Although the greater wax moth, *G. mellonella* has become a favorite model host for the study of interactions between pathogens and the insect immune system (Andrejko & Mizerska-Dudka, 2011), its hematopoietic processes are poorly understood and questions concerning hemocyte proliferation, differentiation and mobilization from hematopoietic organs still remain unsolved (Nakahara et al., 2006; İzzetoglu & Karaçalı, 2010). The AO fluorescent method for classifying hemocytes is more precise than the conventional method using bright field and phase contrast microscopy and promises to facilitate our understanding of hemocyte differentiation in larval hematopoietic organs and hemocyte transformations.

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