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Hemolectin expression reveals functional heterogeneity in honey bee (*Apis mellifera*) hemocytes

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ABSTRACT

The identification of molecular markers considerably facilitated the classification and functional analysis of blood cell types. *Apis mellifera* hemocytes have been classified by morphological criteria and lectin binding properties; however, the use of molecular markers has been minimal. Here we describe a monoclonal antibody to a non-phagocytic subpopulation of *A. mellifera* hemocytes and to a constituent of the hemolymph clot. We demonstrate that the antibody identifies the *A. mellifera* hemolectin, a protein carrying human von Willebrand factor homology domains, characteristic of proteins involved in blood coagulation and platelet aggregation in mammals. Hemolectin expressing *A. mellifera* hemocytes contain the protein as cytoplasmic granules and contribute to the formation of a protein matrix, building up around foreign particles. Consequently, hemolectin as a marker molecule reveals a clear functional heterogeneity of hemocytes, allowing for the analytical separation of hemocyte classes, and could promote the molecular identification of hemocyte lineages in *A. mellifera*.

1. Introduction

Insects have inborn structures and mechanisms for defense against infection by parasites and microorganisms. The system is based on evolutionarily conserved signaling pathways and mechanisms, termed innate immunity (Hoffmann et al., 1999; Hultmark, 2003). Wounding, or pathogens entering the body upon septic injury, immediately trigger the activation of proteolytic cascades, leading to coagulation of hemolymph proteins, clot formation and melanization (Cerenius and Söderhäll, 2011; Theopold et al., 2002). The clot prevents the loss of body fluids and stops the spreading of microorganisms into the hemocoel by immobilizing the bacteria at the wound site (Bidla et al., 2005). Microbes and parasites entering the insect's body are recognized by blood cells termed hemocytes, the effector cells of cellular immunity, circulating in the hemolymph or residing as sessile cells in different tissues (Lanot et al., 2001; Lavine and Strand, 2002; Márkus et al., 2009; Zettervall et al., 2004). Recognition of pathogens triggers hemocyte spreading (Gillespie et al., 1997; Williams et al., 2005) which promotes phagocytosis of microbes or encapsulation of larger foreign particles (Eleftherianos et al., 2009).

Insect hemocytes have been classified on the basis of their morphological, histochemical and functional characteristics (Gupta, 1986; Jiravanichpaisal et al., 2006; Lavine and Strand, 2002), as granular cells, plasmatocytes, spherule cells, oenocytoids and prohemocytes. The best studied organism is *Drosophila melanogaster* where three main classes of hemocytes have been described, plasmatocytes, crystal cells and lamellocytes. The plasmatocytes are the phagocytes, but besides engulfing microbes they produce antimicrobial peptides, extracellular matrix proteins and blood clotting components (Goto et al., 2001). In response to parasitic wasp infection special cell types are formed: the lamellocytes in *D. melanogaster* (Rizki and Rizki, 1992), the nematocytes in *Zaprionus indianus* (Kacsoh et al., 2014) and the multinucleated giant hemocytes in spp. of the *ananassae* subgroup of Drosophilidae

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(Márkus et al., 2015). A minor population of the blood cells—called crystal cells in *Drosophila* (Meister, 2004), and named as oenocytoids in *Lepidoptera* (Lavine and Strand 2002)—secrete components of the phenoloxidase cascade and are involved in the melanization reaction. The classification of hemocytes initially relied on morphological criteria (Rizki, 1957). Later, the identification of different hemocyte classes was facilitated by the development of enhancer trap lines (Rodriguez et al., 1996), identification of immune system and response genes, novel mutations causing melanotic tumor formation in *D. melanogaster* (Braun et al., 1997), and by the generation of monoclonal antibody libraries for the different hemocyte classes (Kurucz et al., 2003, 2007a, 2007b; Rus et al., 2006; Vilmos et al., 2004). These antibodies were also instrumental in the identification of molecules involved in regulating the cellular immune response.

Up to now, various methods have been employed in an attempt to identify the hemocyte subtypes of *Apis mellifera*. These included dye-staining methods and lectin binding assays to characterize honey bee hemocytes. Based on histochemical staining, size and morphological features they were classified into several subtypes, as phagocytic granular cells, plasmatocytes, oenocytoids, coagulocytes, binuclear cells, permeabilized cells and prohemocytes (de Graaf et al., 2002; El-Mohandes et al., 2010; Marringa et al., 2014; Negri et al., 2014; Schmid et al., 2008; Van Steenkiste, 1988). Hemocytes were divided into subtypes by morphological features, ultrastructural characteristics and lectin binding properties as plasmatocytes—representing the major population (90%) of hemocytes in the circulation, subdivided to four subtypes P1-P4 on the basis of size—and a minor population of other cells, as prohemocytes, granular cells, oenocytoids and coagulocytes (de Graaf et al., 2002; Van Steenkiste, 1988). Giemsa staining revealed five hemocyte types, as prohaemocytes, plasmatocytes, oenocytoids, granulocytes, coagulocytes and binucleated cells (El-Mohandes et al., 2010). The most abundant type (over 90%) was the plasmatoycte followed by granular cells and coagulocytes. Using the combination of flow cytometry and microscopy—hemocyte profiling—concluded that the *A. mellifera* hemolymph contains permeabilized cells, plasmatocytes and acellular objects that resemble microparticles (Marringa et al., 2014). Studies (Negri et al., 2014) using hemocytes of L5 stage *A. mellifera* larvae and newly emerged adult workers in *in vitro* experiments identified two cell types in the larva, and four cell types in the adult by their attachment and spreading on solid substrates, agglomeration and phagocytosis. These data all represent of high relevance with respect to the homeostasis of the organism and cellular immunity to parasites and microbes, however, so far the use of molecular markers, including cell-type specific antibodies, for definition of honey bee hemocyte subsets has been minimal.

To address the issue of molecular and functional heterogeneity in honey bee hemocytes we took a combined immunological and functional approach in the hope that development of antibodies with a high specificity for functionally different hemocyte subsets will put cell typing on firm ground (de Graaf et al., 2002; Negri et al., 2015). Using the hybridoma technology in combination with functional assays we aimed to resolve functional heterogeneity of hemocytes on the basis of the expression of a hemocyte specific antigen. We show that the use of a monoclonal antibody combined with functional assays is an effective way to identify different hemocyte subsets in the honey bee.

2. Materials and methods

2.1. Laboratory animals, collection of hemocytes and hemolymph

Studies did not involve endangered or protected species. *Apis mellifera* worker adults and worker larvae were collected from colonies in an apiary in the Szeged-region (Hungary). None of the colonies or the experimental individuals showed symptoms of disease. Fourth-fifth stage worker larvae (L4-L5) were washed off from the brood with *Drosophila* Ringer's solution (130 mM NaCl, 5.0 mM KCl, 1.0 mM $CaCl_2$) and kept between 32 and 35 °C for up to 2 h until hemocyte sampling. The larvae and CO_2-anesthetized young adults were perfused with 100 μl of Schneider's insect medium supplemented with 2 mM L-glutamine (Sigma-Aldrich), 5% fetal bovine serum (FBS; GIBCO) and 0.01% 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich) and the hemolymph was collected in 1.5 ml Eppendorf tubes on ice. The hemocytes were counted in a hemocytometer.

2.2. Production and screening of monoclonal antibodies

Monoclonal antibodies (mAb) (Köhler and Milstein, 1975, 1976) were raised against *A. mellifera* hemocytes as described previously for *D. melanogaster* (Kurucz et al., 2007b) and *D. ananassae* (Márkus et al., 2015). Briefly, BALB/C mice were immunized three times with 10^6 hemocytes in 1 ml *Drosophila* Ringer's solution, with three week intervals. Three days after the final boost spleen cells were fused with SP2/0 myeloma cells by using polyethylene glycol (PEG 1540, Sigma-Aldrich) and the fusion product was distributed in 96 well flat bottomed plates (Costar). Hybridoma culture supernatants were screened by immunohistochemistry on glass-adhered acetone fixed larval and adult hemocytes. The selected hybridomas were subcloned by limiting dilution. For the present study the 4E1, an IgG1κ antibody was used. The isotype of the antibody was determined by IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche Diagnostics).

2.3. Immunohistochemistry (IH) and indirect immunofluorescence (IIF)

All procedures were carried out at room temperature. Hemocytes in Schneider's medium (60 μl) supplemented with 5% FBS and PTU were left to adhere on 12-spot microscope slides (Hendley-Essex: SM-011) for 60 min then fixed with acetone for 6 min, air dried, rehydrated and blocked with PBS (1.78 mM Na_2HPO_4, 5.1 mM Na_2HPO_4, 140 mM NaCl) containing 0,1% BSA (Roche) (PBS-BSA) for 20 min. Samples were incubated with the hybridoma culture supernatant for 1 h, washed three times for 5 min each, with PBS. For immunohistochemistry the samples were incubated with biotinylated goat anti-mouse antibody (1.46 mg/ml diluted 1:500 in PBS-BSA, DAKO) for 45 min, washed three times with PBS. Then the samples were incubated with Streptavidin HRPO (0.85 mg/ml diluted 1:300 in PBS-BSA, DAKO) and washed again three times with PBS. The samples were washed with sodium-acetate solution, (0.2 M, pH 4.6) three times, 3 min each and the reaction was visualized by 0.05% 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich). For visualization of the nuclei DAPI (Sigma 1:400) was used. For indirect fluorescence, as secondary antibodies, Alexa Fluor 568 or Alexa Flour 488 conjugated anti-mouse IgG (Invitrogen, 1:1000) were applied in PBS-BSA for 45 min, containing DAPI. The slides were washed three times with PBS and mounted in Fluoromount G (Southern Biotech). Live hemocytes were reacted with 4E1 antibody and Alexa Flour 568 conjugated anti-mouse IgG (Invitrogen, 1:1000) on ice. Larval cuticle and midgut were dissected in Schneider's medium on ice, fixed with acetone and reacted with the antibodies as described for hemocytes. Samples were examined with an epifluorescence microscope (Zeiss Axioskop 2 MOT) or an Olympus FV1000 confocal LSM microscope.