The cell-mediated immunity of the honey bee

Ph.D. thesis summary

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Introduction

The honeybee *(Apis mellifera)* is a eusocial insect which belongs to the *Hymenoptera* ordo and lives in highly structured colonies. The bees are vital in producing bee products, which are used in food industry, pharmaceutics and cosmetics, and important in maintaining biodiversity. Therefore, diseases of the honey bee cause great economic and ecological losses. To find a solution for the multifactorial phenomenon of colony losses it is necessary to understand the immune defense of the honey bee which is a major factor contributing to fitness of the individuals and of the colony.

Similarly to other social insects, honey bee has both communal barriers and individual protection against parasites and pathogenic microbes. Hygienic behavior, grooming and hive fever are communal defense mechanisms. The individual protection involves a physical barrier, the cuticle, and the reaction of the immune system. The immune response consists of humoral immunity and the cell-mediated arms. Compared to the elements of the humoral responses, the mechanism of cell mediated immunity is much less understood. The cell-mediated immune reactions include the phagocytosis of microorganisms, the encapsulation of larger invaders, the coagulation and clotting of the haemolymph after wounding. The effector cells of the cellular immunity are the blood cells, so called hemocytes. So far, hemocytes were identified by morphological criteria and their lectin binding properties, but due to the lack of molecular markers for these cells the results are controversial, and the mechanisms of cellular immunity in honeybees are not yet studied in details. A more specific method would be necessary to follow the function and differentiation of the blood cell types, and to reveal the hematopoiesis of the honey bee.

Aims

The multifactorial phenomenon of the colony losses generated by biotic and abiotic factors are causing great ecological and economical losses worldwide. In order to reduce this damage it is necessary to understand the structure and the machinery of honeybee immunity. The alternative defense strategies and the humoral components of the individual immune system were identified in details; however, our knowledge of the cell mediated immunity of the honey bee is far from complete, so, the aims of our research are:

1. a more in-depth definition of hemocyte subtypes in the honey bee by developing a so far unique monoclonal antibody-based toolkit to study the cellular components of the honey bee immune system,

2. to classify and characterize the blood cell populations of the larva and the adult by the expression of blood cells molecular markers with the aid of the developed monoclonal antibodies,

3. to study the hemocyte types in the worker queen and drone castes, in the larval and adult developmental stages and to study the function of the different cell types,

4. detailed molecular and genetic analysis of the identified immunological markers,

5. to investigate the elements of the first mechanical defense barrier in the honey bee; the cuticular Vajk proteins previously identified in *D. melanogaster*.

Methods

1. Preparation of hemocyte samples
2. Hybridoma production
3. Immunofluorescent staining
4. Immunohistochemical staining
5. Flow cytometric analysis of fixated, permeabilized hemocytes
6. FITC labeling of bacteria
7. Phagocytosis assay
8. Coagulation assay
9. Encapsulation assay
10. Immunoprecipitation
11. Western blot assay
12. Silver staining
13. Preparation of double stranded RNA and RNA interference
14. Statistical analysis
15. Microscopic analysis

Summary of the results

1. We developed a toolkit for the characterization and classification of the honeybee hemocytes by cell-type specific antigens. In order to find molecular markers for functional subsets of blood cells, first, we raised a set of monoclonal antibodies to larval and adult hemocytes, similarly to those developed in our laboratory to *Drosophila* species. We identified marker molecules by their expression pattern with immunofluorescence and immunohistochemical methods. We identified markers expressed by all cell types (pan-hemocyte markers), markers, specific for the plasmatocytes (plasmatocyte markers), for the oenocytes (oenocyte markers) and for the granulocytes and oenocytes (granulocyte-oenocyte markers). With the defined marker panel we tagged the three main classes of hemocytes: the plasmatocytes, the granulocytes and the oenocytes.

2. We have identified the proportion of the different blood cell types in the larval and adult developmental stages. In the larvae 12%-23% of the blood cells were plasmatocyte, 87%-76% were granulocyte from the L1 to the L5 stages, in the young adults 77% were plasmatocyte, 22% were granulocyte and in the older adults 51% of the blood cells were plasmatocyte, 48% were granulocyte. In the studied developmental stages the rate of the oenocytes was 1%. The proportion of the plasmatocyte marker positive cells is increasing through the development until the individuals reach the adult stage, in the older adults the expression is decreased. The granulocyte-oenocyte marker is expressed in all hemocyte types in the larva, but it is specific for the granulocytes and the oenocytes in the adult. With the aid of the developed monoclonal antibodies we can follow the changes in the marker expression of the different hemocyte types through the development.

3. We analyzed the adults of the queen and drone casts too. The markers expression on the hemocyte types of the queens and drones were similar to that of the workers. It may be anticipated, that the cellular immune responses are the same in all casts.

4. In previous studies of other laboratories the classification by flow cytometric analysis of the different honey bee hemocyte types was not sufficient. With the produced

monoclonal antibodies we have identified cytoplasmic molecular markers specific for the blood cell populations, which can be used in flow cytometric analysis to follow the changes in the proportion of the different hemocyte types.

5. With the combination of a defined marker panel and functional tests for the cell-mediated immune responses we observed, that the granulocytes take up microorganisms by phagocytosis. The plasmatocytes form cell-aggregates to isolate foreign particles in the body cavity, the aggregates express the plasmatocyte and the oenocyte markers. In the *ex vivo* clot formation assay the plasmatocytes built up a fiber matrix in the coagulum, which expresses the plasmatocyte and the granulocyte-oenocyte markers.

6. The oenocyte markers are expressed in *D. melanogaster* crystal cells and in a subpopulation of the lamellocytes which produce the prophenoloxidases (PPOs) in the encapsulation response and in wound healing. The molecular mass of the protein reacting with the 2.28 oenocyte specific antibody is ~75 kDa and ~150 kDa. We presume that the detected ~150 kDa molecular mass band is the dimeric form of the ~75 kDa band. In previous studies the *A. mellifera* PPO (AmPPO) was identified in the haemolymph and the molecular mass of the activated enzyme is 74.4 kDa, which is similar to our results. We compared the proteins sequences of the three DmPPOs and the AmPPO and found 47%-60% sequence homology. According to these results we postulate that the protein expressing the 2.28 marker is the AmPPO.

7. The result of the immunoprecipitation of the 4E1 protein followed by an LC-MS/MS analysis showed that the identified protein is the *A. mellifera* hemolectin (AmHml). To challenge the results we did an RNA interference experiment. In the RNAi the proportion of the 4E1 marker positive cells, was lower than the control samples confirming that the 4E1 protein is the *A. mellifera* hemolectin.

8. By using the AmHml antibody we marked the blood cells of the honey bees kept in neonicotinoid treated sunflower, bees treated with amitraz, and the differences between *Varroa* sensitive (VSH) versus non VSH bee lines and mite infected versus non-infected individuals. We did not record significant changes in the ratio of the plasmatocytes in the hemolymph.

9. In the functional tests of the hemocytes of naïve adults we observed that 22% of the hemocytes are phagocytic granulocytes and after immune induction (sterile wounding, *Varroa* infection, chemical treatment, and foreign macroparticles in the body cavity) we did not observed alteration in the proportion of the hemocytes or the differentiation of new effector blood cell types.

10. After we analyzed the cellular immunity of the honey bee we have also studied the first line of the defense: protection of the organism from the external mechanical damages. In insects the cuticle is responsible for this function. We investigated the expression of Vajk proteins which are expressed in the cuticle of *D. melanogaster* pupae and have been predicted to be expressed in the honey bee. We showed the presence of the Vajk 4 protein in honey bee pupae by Western blot analysis.

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