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Functional differentiation of spider hemocytes by light and transmission electron microscopy, and MALDI-MS-imaging

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ABSTRACT

The most abundant cell types in the hemolymph of *Cupiennius salei* are plasmatocytes (70–80%) and granulocytes (20–30%). Both cells differ in shape, cytochemical and transmission electron microscopy staining of their cytoplasma and granules. According to MALDI-IMS (matrix-assisted laser desorption ionisation-mass spectrometry imaging), granulocytes exhibit ctenidin 1 (9510 Da) and ctenidin 3 (9568 Da), SIBD-1 (8675 Da), and unknown peptides with masses of 2207 and 6239 Da. Plasmatocytes exhibit mainly a mass of 6908 Da. Unknown peptides with masses of 1546 and 1960 Da were detected in plasmatocytes and granulocytes. Transmission electron microscopy confirms the presence of two compounds in one granule and cytochemical staining (light microscopy) tends to support this view. Two further hemocyte types (cyanocytes containing hemocyanin and prehemocytes as stem cells) are only rarely detected in the hemolymph. These four hemocyte types constitute the cellular part of the spider immune system and this is discussed in view of arachnid hemocyte evolution.

1. Introduction

The immune system of invertebrates is an innate immune system, relying on cellular reactions (such as phagocytosis, encapsulation, and nodulation) and on humoral responses (pathogen recognition and elimination). Important are hemolymph coagulation, clotting/proteolytic cascades with a variety of elements, and antimicrobially acting compounds (Cerenius and Söderhäll, 2011; Cerenius et al., 2010a).

In contrast to insects and crustaceans, for most groups of chelicerates the immune system is not well-investigated. Moreover, most studies are limited to Merostomata, mainly the xiphosuran horseshoe crabs *Limulus* and *Tachypleus*, a species-poor marine group (Kawabata, 2010). Additionally, research on tick innate immunity and their defence against microbial invaders resulted in the identification of a variety of antimicrobially acting compounds (Kopacek et al., 2010). However, other large arachnid sister groups are much less analysed. This is especially true for ecologically important and species-rich taxa such as scorpions and spiders, which contain many species of medical importance.

Central aspects of the immune system in arachnids, such as (1) location of the hematopoietic tissue, (2) number and kind of hemocyte types, and (3) main elements of the humoral response are astonishingly differing among the various arachnid groups, making extrapolations and prognoses very difficult.

1) In some crustaceans (crayfish) the hematopoietic tissue, where hemocytes are produced from stem cells, is a tissue sheet, covering the dorsal and dorsolateral sides of stomach and ophthalmic artery (Noonin et al., 2012), for the penaid shrimps, this tissue is located on the dorsolateral surface of the foregut in paired nodules. However, some penaid shrimps possess in addition hematopoetic tissue at the base of the maxillipeds, e.g., close to the antennal artery (Cerenius et al., 2010b). In xiphosurans and ticks the location of hematopoetic tissue is unknown (Kopacek, personal communication). In scorpions, this tissue is concentrated in supraneural and lateral lymphoid glands, close to the ventral supraneural artery (Farley, 1984), whereas in spiders the hematopoietic tissue is situated in the heart wall of the dorsal aorta vessel as a diffuse system of stem cells (Franz, 1904).

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2) There is a confusing diversity of hemocyte types, which can be distinguished among major arachnid taxa. Depending on the microscopic observation technique, the intensity of cell granulation and ultrastructural characteristics, a variety of cell types has been described. Often, the physiological status of the animal (e.g., pre- or post-moulting, healthy or infected), rapid cell transformation during the hemolymph collecting process and/or maturation and secretion processes in hemocytes were neglected. For ticks, (Borovickova and Hypsa, 2005) describe prohemocytes and 3 or 4 hemocyte types, depending on species. For spiders, (Sherman, 1981) summarises four categories of different hemocyte types depending on spider species.
3) Knowledge of components of the humoral response in arachnids is even more diffuse. Plenty of information is available for xiphosurans (Kawabata, 2010), leading to the assumption that coagulation of hemolymph and melanisation, after inducing phenoloxidase activity of hemocyanin, constitute central elements. However, this cannot be generalised for all arachnids, since the coagulation cascade of tick hemolymph has not yet been investigated (Kopacek et al., 2010) and ticks do not possess hemocyanin, which would lead to melanisation (Kopacek et al., 2010). Both elements exist in spiders (Kuhn-Nentwig and Nentwig, 2013). Additionally, spiders rely on hemocyanin fragments active against certain microbial pathogens (Riciluca et al., 2012).

It is evident that a further approach to better understands the immune system of spiders has to overcome the stage of discussing the number of hemocyte types, especially since this was so far restricted to morphological issues, whereas histochemical differentiation and functional aspects were largely neglected. For horseshoe crabs, more than 99% of all hemocytes are granulocytes including large and small granula (Kawabata, 2010), thus indicating some kind of division of work between the different granule types. Cyanocytes, responsible for hemocyanin synthesis (Fahrenbach, 1970) and non-granular blood cells (Shakibazadeh et al., 2013) seem to cover less than 1% of all hemocytes.

For ticks, Borovickova and Hypsa (2005) included phagocytic activity into their cell type differentiation scheme and Kopacek et al. (2010) showed the expression of several humoral response components in hemocytes but no attribution to a given cell type was possible. For spiders, several antimicrobial peptides are known from hemocytes: gomesin (Silva et al., 2000), defensins (Baumann et al., 2010a), glycine-rich peptides (Baumann et al., 2010b; Lorenzini et al., 2003), and an antifungal hemocyanin fragment (Riciluca et al., 2012). Also a single insulin-like growth factor binding domain protein (SIBD-1) was isolated and is obviously involved into regulatory processes of the immune defence (Kuhn-Nentwig et al., 2011).

Considerable investigations on hemocytes of the spider *Cupiennius salei* (*C. salei*) were performed by Seitz (1972, 1976) but no immunological function was attributed. In 1981, Sherman reviewed all hemocyte literature concerning mygalomorph and araneomorph spiders (Sherman, 1981). Here, we differentiate the diversity of hemocytes in *C. salei*, meanwhile the best investigated spider species (Barth, 2001) and link two hemocyte types to masses of produced compounds in the respective hemocytes. We have done this with a combination of light microscopic cell type analysis and matrix-assisted laser desorption ionisation mass spectrometry imaging (MALDI-IMS), a promising approach (Lynn et al., 1999; Rubakhin et al., 2000), which has so far not yet been used for such a purpose. Our results give new insights into the main structural organisation of the immune system of spiders.

2. Materials and methods

2.1. Spider stock and hemolymph collection

Adult and healthy females (4–12 weeks after the last moulting) of *C. salei* were obtained from our permanent breeding line and hemolymph collection was carried out as described (Baumann et al., 2010b).

2.2. Light microscopic characterisation of hemocytes

Type and ratio determination of different hemocytes was done immediately after hemolymph collection. The hemoylmph was diluted (1:1) with sodium citrate buffer (30 mM trisodium citrate, 26 mM citric acid, 0.45 M NaCl, 0.1 M glucose, 10 mM EDTA, pH 4.6) (Söderhäll and Smith, 1983) to prevent coagulation. For light microscopic cell type analysis on a Zeiss Axioplan 2, (400× magnification) 10 μl of diluted hemolymph from 5 spiders were spread on glass slides. Altogether, for each spider 20 fields of view were analysed and the cell type ratio was determined for 3188 cells.

Size determination of hemocytes was carried out with short-term hemocyte cultures as described elsewhere (Ballarin et al., 1994). Briefly, to avoid shrinkage of cells, 10 μl of hemolymph dilution was placed in the center of the culture chamber (plastic film rings of 12 mm diameter, which were glued to the glass slide and coverslips using vaseline) and kept upside down for 30 min. After adhesion of hemocytes to the coverslips, cells were quantified by light microscopy as described above. Cell length and width were measured of 768 plasmatocytes, 675 granulocytes, 2 cyanocytes and 2 prohemocytes.

Staining air dried hemolymph smear on glass slides (10 μl of a 1:1 dilution with sodium citrate buffer, see above) with Giemsa's dye (Merck, Germany) was done according to the producer's manual after fixation in methanol for 3 min.

2.3. Transmission electron microscopic characterisation of hemocytes

After hemolymph collection, hemolymph of three adult female spiders were diluted 1:1 with sodium citrate buffer and hemocytes separated from plasma by centrifugation at 800*g*, for 10 min at 4 °C. Hemocytes were washed twice with sodium citrate buffer and again centrifuged. The pellets were submerged with fixative, which was prepared as follows: 2.5% glutaraldehyde in 0.15 M HEPES with an osmolarity of 709 mOsm and adjusted to pH 7.3.

Cells remained in the fixative at 4 °C for at least 24 h before being further processed. Cells were then washed with 0.15 M HEPES twice for 5 min, post-fixed with 1% OsO_4 in 0.1 M Na-cacodylate-buffer at 4 °C for 1 h, washed with 0.05 M maleat-NaOH buffer three times for 5 min, and then block stained in 0.5% uranyl acetate in 0.05 M maleat-NaOH buffer at 4 °C for 1 h. Afterwards, cells were washed in 0.05 M maleat-NaOH buffer three times for 5 min and dehydrated in 70%, 80%, and 96% ethanol for 15 min in each case at room temperature. Subsequently, cells were immersed in 100% ethanol three times for 10 min, in acetone twice for 10 min, and finally in acetone-epon (1:1) overnight at room temperature. The next day, cells were embedded in epon (Fluka) and left to harden at 60 °C for 5 days.

Sections were produced with an ultramicrotome UC6 (Leica Microsystems), first semi thin sections (1 μm) for light microscopy, which were stained with a solution of 0.5% toluidine blue O (Merck), and then ultrathin sections (70–80 nm) for transmission electron microscopy. Sections, mounted on 200 mesh copper grids, were stained with uranyl acetate and lead citrate with an ultrostainer (Leica Microsystems). Sections were examined with a