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Direct comparison between *in vivo* and *in vitro* microsized particle phagocytosis assays in *Drosophila melanogaster*

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ABSTRACT

The effects of micro and nanoparticles on the innate immune system have been widely investigated and a general lack of agreement between *in vivo* and *in vitro* assays has been observed. In order to determine the origin of these discrepancies, there is a need for comparing the results of *in vivo* and *in vitro* phagocytosis assays obtained using the same particles and same immune cells. Here, we establish an *in vivo* polystyrene microsized particle phagocytosis assay in *Drosophila melanogaster* and compare it with an *in vitro* assay consisting of exposing the same immune cells in culture to the same particles. The distribution of number of phagocytized beads per cell was shifted to lower numbers of beads per cell in the case of the *in vitro* assay compared to the *in vivo* assay, which we suggest is partly due to a reduced amount of membrane available in cultured cells.

1. Introduction

Micro and nanoparticles are omnipresent in our daily life. They can be found for instance in food, paints and cosmetics. It is therefore crucial to evaluate their effects on the ecosystem as well as on humans. In the literature, the most evaluated parameters are biodistribution, bioaccumulation and innate immune system activation such as degree of phagocytosis by macrophages. Whereas biodistribution and bioaccumulation can only be assessed using *in vivo* systems (through direct imaging or sectioning before imaging), assessing the effects of particles on the innate immune system can be achieved in three ways: *in vivo*, *ex vivo* and *in vitro*.

In vivo assays usually involve post-exposure tissue sectioning and subsequent electronic or optical microscopy to assess the internalization of particles in immune cells (Gallentoft et al., 2015; Linsmeier et al., 2009; Principi et al., 2016; Ryman-Rasmussen et al., 2009). In the case of inhalation exposures, the analysis of the number of neutrophils in the bronchoalveolar fluid of, for instance, rodents, has been shown to give indications of the inflammatory nature of particles (Poulsen et al., 2016; Wallin et al., 2016).

In *ex vivo* studies, organs are dissected and cultured in the presence of particles (Hofmann et al., 2015; Papageorgiou et al., 2014). This type of assay represents a good compromise between cell culture studies, which are not always representative of the *in vivo* situation, and the expensive and often cumbersome *in vivo* studies. Alternatively, organ-like structures can be assembled *in vitro* from various cells, which reproduce the key functions of the real organ, and which can be used for toxicity studies (Esch et al., 2014; Huh et al., 2010).

In vitro cultures of immune cells have the advantage of being cheap, enabling a better controlled particle exposure and enabling faster readout, without any dissection and sectioning required. However, as mentioned above, *in vitro* studies can possibly differ from the *in vivo* situation. For instance, they cannot accurately assess the recruitment/synthesis of new immune cells, and can therefore possibly report particle concentration in immune cells that would not occur *in vivo* (Cohen et al., 2014). Moreover, the effects of extracellular environment are different *in vitro* from the *in vivo* situation. For instance, the fact that cultured cells adhere on a flat substrate can influence both the activation of immune cells and their phagocytic capacity (Blakney et al., 2012; Cohen and Cline, 1971; Patel et al., 2012; Schinwald and Donaldson, 2012). To address these issues, some studies have proposed to culture immune cells on soft substrates (Sridharan et al., 2015). However, the influence of the extracellular environment such as protein and other cell secretion remain different *in vitro* from the *in vivo* situation.

In order to benefit of the advantages of the *in vitro* systems, we need to be aware of their limitations. Therefore, it is necessary to compare *in vivo* and *in vitro* experiments performed on identical immune cells and

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particles.

Drosophila melanogaster is a cheap and "easy-to-maintain" organism that can be used for both *in vivo* and *in vitro* phagocytosis studies (Rämet et al., 2002; Ulvila et al., 2011). It enables *in vivo* assessment of particle phagocytosis through injecting particles in the hemolymph of larvae, bleeding the larvae and imaging the extracted hemocytes (immune cells) and particles. The hemocytes of *Drosophila* can also be cultured *in vitro* where they can be exposed to particles and imaged.

Here, we use *Drosophila* and polystyrene microsized particles (also referred to PS beads in this paper) to directly compare key aspects of particle phagocytosis assessed using both *in vitro* and *in vivo* experiments. PS beads were either injected in the living fly hemolymph or added to a culture of *Drosophila* hemocytes. In both cases, hemocytes were allowed to incubate with the beads for 4 h, after which the bead distribution was investigated using optical fluorescence microscopy and scanning electron microscopy (SEM).

The results show that the *in vivo* phagocytic capacity is governed by the total surface area of phagocytized beads. At bead concentrations where the hemocytes are saturated, *in vitro* assays show a significantly lower number of phagocytised beads than for the *in vivo* assay. This may be explained by the fact that a large part of the cultured cell surface is immobilized by substrate adhesion. These results highlight the bias introduced by the presence of a substrate in the *in vitro* situation.

2. Materials and methods

2.1. PS beads

PS beads with diameters of 1, 3, 5, 7 and 10 μm were purchased from Spherotech, Inc. and in all experiments, non-functionalized fluorescent PS beads were used. The beads were provided as 1% weight per volume (w/v) suspensions in deionized water with 0.2% sodium azide to prevent bacterial growth. PS bead suspensions with concentrations of 1%, 10%, 20% and 30% w/v in PBS, were produced by centrifuging and re-suspending in filtered PBS to the respective concentration. The beads were subsequently washed three times by centrifuging and re-suspending the beads in filtered PBS. For determining the bead concentration, each suspension was further diluted in PBS to a nominal 5000 beads/μl concentration. A 0.1 μl drop of the diluted bead suspension was subsequently dried on a glass slide and all beads where counted using dark field optical microscopy. This was repeated three times for each suspension. Deviation of > 50 beads from the expected 500 beads (> 10% deviation) was compensated, either by further dilution or by centrifugation followed by resuspension in a smaller volume of PBS. The concentration after compensation was reevaluated until determined to be within the tolerated range (< 10% deviation). The suspensions were sonicated for 30 s just before injection.

The bead size-distribution was determined using dark field optical microscopy and the particle analyzer function in ImageJ (Abràmoff et al., 2004). The zeta potential of the PS beads was determined with 5 μm beads in PBS using a Malvern Zetasizer Nano ZS™. See *Supporting information* for final concentrations, bead size distributions and zeta potential.

2.2. Flies

For all experiments, the flies used were Hemese-Gal4, UAS-GFP (kind donation by Dan Hultmark), which exhibits hemocyte specific GFP-expression (Zettervall et al., 2004). Flies were kept at 25 °C on top of standard fly food and on a 12 h night/day cycle.

2.3. In vivo injections

For the injections, a heat-pulled micro capillary needle, beveled at a 30° angle was used. The needle was connected to an over pressure and injections were controlled with a foot pedal. The injected volume was determined by measuring the column height in a glass micro capillary. The injection site, in the posterolateral region, was used consistently and special care was taken to avoid rupturing the intestine. In all experiments, 50 nl of bead suspension were injected directly into the body cavity of third instar larvae. All experiments were carried out 3 times.

2.4. Quantification of phagocytosis using optical microscopy

The slides with hemocytes and beads (obtained both using *in vivo* and *in vitro* experiments, see below) were studied using fluorescence microscopy with a 63 × oil immersion objective (NA = 1.40) in combination with differential interference contrast (DIC) microscopy to assess the extent of phagocytosis. Phagocytosis was quantified by counting the number of beads inside individual hemocytes as well as the number of beads outside of the cells. A minimum of 100 cells were counted for each slide.

2.5. SEM investigations of bead internalization

Samples were prepared for SEM investigations by drying the samples in ethanol dilution series, followed by sputtering (Polaron E5100) a 10 nm layer of platinum. Using SEM (LEO 1560 with Thermal field emitter source at 10 kV accelerating voltage and an InLens detector at 30° sample tilt), the number of bound beads for each cell was counted and among these, the number of internalized, as well as non-internalized was evaluated.

2.6. In vivo assessment of the bead size that can be phagocytized by Drosophila hemocytes

We injected beads (see Section 2.3. *In vivo* injections) of different sizes (1, 3, 5, 7 and 10 μm) at a concentration of 1% (w/v) in PBS. The larval hemolymph, containing the hemocytes, was collected and studied 4 h post injection, by bleeding 10 larvae on a glass slide containing 50 μl of PBS. The extent of phagocytosis was quantified using optical microscopy (see Section 2.4. Quantification of phagocytosis using optical microscopy).

2.7. Phagocytic capacity

We studied the cellular limits of phagocytosis, by injecting beads (see Section 2.3. *In vivo* injections) of two different sizes (3- and 5 μm, in two separate sets of experiments) at a high enough concentration to saturate the hemocytes with PS beads. After injecting a range of concentrations (1 to 30% w/v), it was determined that 20% w/v was enough to saturate the hemocytes with beads, and this concentration was subsequently used to evaluate the phagocytic capacity. The extent of phagocytosis was quantified using optical microscopy (see Section 2.4. Quantification of phagocytosis using optical microscopy).

2.8. In vitro phagocytosis assay

We collected the hemolymph from 10 third instar larvae directly on a cover glass containing a 100 μl drop of culture medium, and let the hemocytes attach to the cover glass for 1 h. The hemocytes were subsequently exposed to 5 μm beads with the same bead to cell ratio as in the injection experiments (corresponding to 50 nl of 20% w/v for each larva, see Section 2.7. Phagocytic capacity). The hemocytes were then incubated with the beads for 4 h. The culture of primary *Drosophila* hemocytes was carried out using an established protocol (Sampson and Williams, 2012). The cell culture media consisted of 75% (v/v) filtered Schneider's insect medium supplemented with 25% (v/v) fetal bovine serum (FBS), and the cells were kept at 25 °C. After 4 h of culturing, the extent of phagocytosis was quantified using optical microscopy and SEM (methods described above).