

## ORIGINAL ARTICLE

# Phagocytosis of insect haemocytes as a new alternative model

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### Summary

Phagocytosis is an important function of both insect haemocytes and mammalian blood cells. Linden bugs and cotton leaf worms have been suggested as new alternative models for ecological and drug toxicology but no data on their haemocyte physiology have been published. Our assays with particle ingestion of the NBT test were carried out on prohaemocytes, granulocytes, plasmatocytes and spherulocytes of adult linden bug and cotton leaf worm larvae. We found that phagocytic activity is on average 10% in the linden bug, and 50% in cotton leaf worm haemocytes: the phagocytic index is 3.5 in both species and nitroblue tetrazolium reduction is 0.5 in the linden bug and 3.2 in the cotton leaf worm. Phagocytic characteristics of the prohaemocytes and granulocytes in the cotton leaf worm are closed to mammalian neutrophil physiology. Our data suggest that cotton leaf worm haemocytes may be a new potential alternative model for screening of phagocytosis.

**Key words:** alternative model; phagocytosis; particle ingestion; NBT; linden bug; cotton leaf worm

## INTRODUCTION

Studies of phagocytosis in various mammalian species focusing on the clinical or toxicological background revealed a wide diversity depending on the dominant environment. For example, the granulocytes of rats, with a higher infection risk, have higher phagocytic potential than human granulocytes (Větvička et al. 1982, Berger 1988).

The most frequent examinations of phagocytosis in mammalian haematotoxicology cover the ingestion

of various particles (phagocytic activity and index) and the production of reactive oxygen species as part of the oxidative killing of invading pathogens (Wang and Schwarz 1959, Sanchez et al. 2010, Pohanka et al. 2011, Steevels and Meyaard 2011). Phagocytic activity and index reflect the first stage of phagocytosis. There are various particles which can be used as yeast cells, *Escherichia coli*, cadmium and HEMA particles. The examination of oxidative killing reflects the last phase, evaluated by the nitro blue tetrazolium reduction test (NBT; Johnston and Baehner 1971).

Insect haemocytes participate in immunity reactions (Strand 2008). The innate cellular defences against infection include haemocyte-mediated responses like phagocytosis, microaggregation, nodulation and encapsulation (Stanley and Miller 2006, Marmaras and Lampropoulou 2009). Phagocytosis is an evolutionarily conserved cellular response in all eukaryotic phyla (Yutin et al. 2009) but most of the available data refer to mammalian

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circulating white blood cells and macrophages. Too little is known about phagocytosis in insects as it is difficult to conduct experiments on insect haemocytes and identify these cells in some species.

Both linden bug (*Pyrrhocoris apterus*) adults and cotton leaf worm (*Spodoptera littoralis*) larvae are used as “laboratory” insects in physiology research, and their insectaria are standardized in ways similar to the more well-known rodent vivaria (Berger 2009a, Picmonova and Berger 2012). Their haemocyte morphology has been described and these species are suggested as applicable biomodels for toxicological screening elsewhere (Gelbič et al. 2006, Berger and Slavičková 2008, Berger 2009b, 2010). Nevertheless, the functional characteristics of haemocytes in these two insect species are unknown. The aim of this study is to evaluate them as potential alternative models for the screening of phagocytosis *in vivo* using methods which are standardized in mammalian physiology.

## MATERIAL AND METHODS

### *Insects*

We used both males and females of the adult linden bug, *Pyrrhocoris apterus* (L.) (Heteroptera), and the larvae of the cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera), in the last instar. Insects were kept at a constant temperature of  $25 \pm 1$  °C, in  $75\% \pm 10\%$  relative humidity under an artificial photoperiod of 16 h of light and 8 h of dark.

Adults of the linden bug were fed with lime seeds, the larvae of cotton leaf worm with an artificial Stonefly *Heliothis* diet (prepared by Wards, Rochester, USA). The haemolymph of the linden bug was drawn after cutting off the distal part of an antenna; that of the cotton leaf worm after entomological pin injury.

### *Determination of phagocytosis*

In our pilot study prior to this, we observed that a mixture of haemolymph with tested particles blackens, so that the phagocytosis cannot be investigated. Evaluating the stability of haemocytes in Grace's medium we found that the blackening was eliminated for microscopical examinations but that the granulocytes decay within a few minutes, although plasmatocytes were the most resistant (Burešová and Berger 2002). In the present paper, we used Ringer solution to prevent both the blackening and the destruction of cells during the incubation times used.

A suspension of 2.5 µl HEMA particles (radiation copolymer of 2-hydroxyethylmethacrylate manu-

factured by Artim Prague, Czech Republic, diameter 1.2 µm), was incubated in 15 µl of the Ringer solution and 5 µl of haemolymph for 20 minutes at 37 °C (Berger 1988). Smears were air-dried and then stained using the Pappenheim panoptic method. At least 100 haemocytes from each animal were examined (Nikon Eclipse 50i, Plan Apo 100x/1.40 oil, immerse oil).

Phagocytic activity is expressed as the ratio between the number of haemocytes with phagocytised particles and the number of all evaluated haemocytes. A phagocytic index is the average of engulfed particles per one phagocytising haemocyte.

The NBT (Pick et al. 1981) was made using 25 µl of the Ringer solution containing 0.1% of nitroblue tetrazolium (NBT, Sigma-Aldrich, Prague, Czech Republic, Cat. No 68H5075) and 25 µl of fresh haemolymph at 37 °C for 20 min. Several samples were used for smears which were immediately fixed in formaldehyde steam for 10 min and then stained by nuclear fast red (Merck, Darmstadt, Germany, Cat. No 8598671). Incubation in a further sample was stopped by 60 µl of 2M KOH and 70 µl of dimethylsulphoxide (Rook et al. 1985). Dark nitroblue formazan was measured in the Sunrise (Tecan Group Ltd., Männedorf, Switzerland) reader at 620 nm against blank samples without a haemolymph.

### *Statistic analysis*

The results are expressed as mean $\pm$ s.e.m. Data were compared using the two-sided Mann-Whitney U test at the significance level  $2\alpha=0.05$ .

## RESULTS

The phagocytic ability of haemocyte populations was evaluated by ingestion of HEMA particles and by the test for the metabolic capacity to kill ingested cells on the model of the reduction of NBT to formazan. Our findings demonstrate that haemocytes of both examined species may have a certain role in defense responses to foreign microorganisms in both species examined.

### *Phagocytic activity and index*

Using linden bug haemocytes, HEMA particles were ingested by prohaemocytes (Fig. 1), granulocytes and plasmatocytes and no statistically significant difference was observed between male and female haemocytes (Table 1). Granulocytes ingest a significantly lower number of particles than prohaemocytes and plasmatocytes. No phagocytosis was observed in the spherulocytes. Comparing data on the ingestion of yeast cells and HEMA particles

Table 1. **Phagocytosis estimated by ingestion in haemocytes.**

	Linden bug		Cotton leaf worm	
	M	F	M	F
Phagocytic activity (%)				
Prohaemocytes	14.9 <sup>a</sup> ±2.8 <sup>b</sup>	12.7±1.9	57.7±6.3	43.9±4.3 <sup>+</sup>
Granulocytes	3.0±2.0*	2.4±1.8*	57.5±3.5	46.9±3.3
Plasmatocytes	9.1±0.8	10.3±1.7	12.7±10.5	25.8±12.3
Spherulocytes	0.0±0.0*	0.0±0.0*	0.0±0.0*	0.0±0.0*
All populations of haemocytes	9.0±0.7	10.4±1.5	57.0±3.7	43.5±3.3 <sup>+</sup>
Phagocytic index				
All	3.21±0.35	3.26±0.28	4.8±0.2	3.0±0.3 <sup>+</sup>
N	10	10	9	10

<sup>a</sup> mean, <sup>b</sup> standard error of mean, \* statistically significant versus All, <sup>+</sup> statistically significant as compared with males

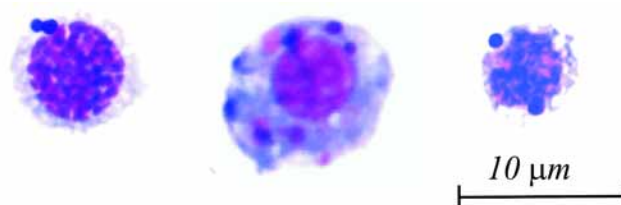


Fig. 1. **Ingestion of HEMA particles and by a cotton leaf worm prohaemocyte (left) and granulocyte (middle) and by a linden bug prohaemocyte (right).**

we can see that the phagocytic activity for prohaemocytes and plasmatocytes was significantly higher using HEMA particles while their ingestion by granulocytes was lower, but statistically not significant.

Phagocytosis was expressed in cotton leaf worm haemocytes at a significantly higher rate compared with linden bug cells (Table 1, phagocytic activity). Although the number of phagocytosing cotton leaf worm haemocytes was 4–5 times higher than linden bug cells, the number of ingested particles per cell, i.e. phagocytic index, was almost equal. It was slightly increased in cotton leaf worm males.

#### *NBT test*

We observed that prohaemocytes, granulocytes and plasmatocytes can reduce nitroblue tetrazolium (Fig. 2) while no spherulocyte with black precipitate was found. We found the maximum of formazan concentration following 2 hrs of linden bug haemocyte incubation, while its peak in cotton leaf worm haemocytes was reached following 8 hr incubation. Values from the cotton leaf worm haemocytes were higher. No significant difference was found between male and female haemocytes in formazan production (Table 2).

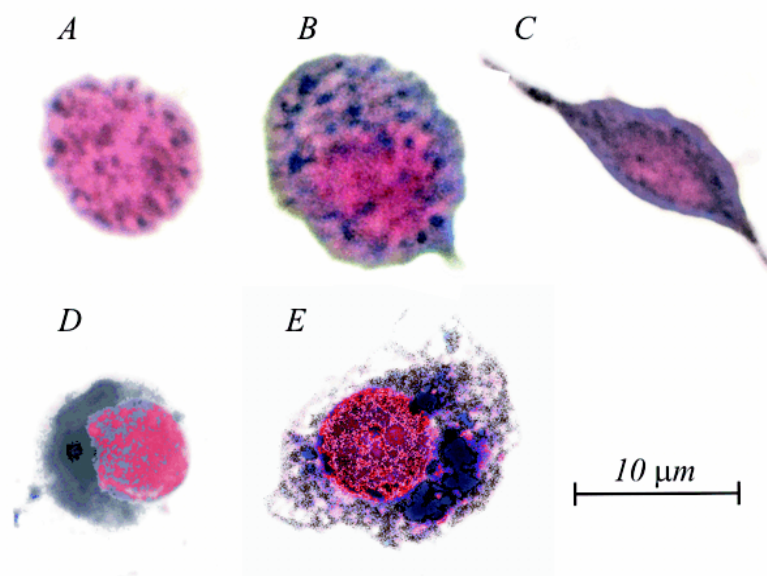


Fig. 2. **Formazan (dark precipitates) in prohaemocyte (A), granulocyte (B) and plasmatocyte (C) of linden bug and in prohaemocyte (D) and granulocyte (E) of cotton leaf worm.**

Table 2. **Nitroblue tetrazolium reduction in all types of haemocytes in NBT test.** Absorbance measured in Elisa reader at 620 nm.

No haemocytes (blank)	Linden bug		Cotton leaf worm	
	M	F	M	F
0.00 <sup>°</sup> ±0.00 <sup>b</sup>	0.52 <sup>a</sup> ±0.03 <sup>b§</sup>	0.53±0.04 <sup>§</sup>	3.21±0.16 <sup>§</sup>	3.10±0.35 <sup>§</sup>

<sup>°</sup> mean of ten samples, <sup>§</sup> statistically significant versus blank samples

Other symbols as in Table 1

## DISCUSSION

The data presented on the phagocytosis and NBT tests suggest that haemocytes in both species evaluated may have a role in defence responses to foreign organisms, although this activity of spherulocytes seems to be inconsiderable. The phagocytic activity of prohaemocytes in both insects was an unexpected finding in contradiction to the data on several other taxa (cf. Hernandez et al. 1999).

Ingestion tested by HEMA particles seems to be better estimated than similar evaluations of mammal phagocytes. We observed lower phagocytic activity in the linden bug while ingestion in cotton leaf worm prohaemocytes and granulocytes was close to that in

mammal neutrophils (Berger 1988). The mean number of ingested particles was lower in the linden bug haemocytes than in mammalian granulocytes while the phagocytic index for cotton leaf worm haemocytes seemed to be similar. Ehlers and co-workers (1992) also showed that ingestion ability depends on the character of the particles used, and that it has a similar influence on methods of phagocytic ability in mammals (cf. Slapničková and Berger 2002). Nevertheless, it seems that HEMA particles are ingested intensively (Berger 1988, Burešová and Berger 2002) and their use could better reveal interspecific variances. The values of phagocytic activity are lower for linden bug haemocytes, compared with both mammalian blood

cells (Berger 1988) and the data presented here on the cotton leaf worm, are close to similar data on other insect species, Wax Moth, *Galleria mellonella* (Wiesner et al. 1996).

Our finding of many positive cells in the NBT test could suggest that a lower phagocytic activity of linden bug haemocytes is apparent, maybe as a result of the faster disintegration of these haemocytes *in vitro*; that is, granulocytes wear off *in vitro* for their degranulation during incubation for ingestion examinations, as we have estimated by differential counts in a previous pilot study (Burešová and Berger 2002) and they could lead to a morphology which is close to plasmatocytes in panoptically stained smears, while granulocytes remain present during the NBT test.

The NBT test reflects the production of superoxide anions (Liochev et al. 1995). We found formazan in the prohaemocytes, granulocytes and plasmatocytes of both linden bug and cotton leaf worm. Formazan formation was also found in the haemocytes of several species (Toru 1994, Glupov et al. 2001). In contrast, Hyršl and co-workers (2004) did not find formazan in the isolated larval haemocytes in silkworm nor did Glupov et al. (2001) in small tortoiseshell.

Different findings of phagocytic characteristics in the haemocytes of various insect species could suggest that the role of phagocytosis in invertebrate species could be less uniform than that in vertebrate blood cells. Another explanation for the inconsistencies in the literature on insect phagocytosis could be the larger diversity in metabolic pathways during phagocytic processes.

We found that the most important characteristics of phagocytosis in both linden bug and cotton leaf worm haemocytes can be examined by methods commonly used in both human and veterinary haematology. We suppose that there could be alternative biomodels applicable to haematotoxicological express assays – so-called screening; for example, cotton leaf worm larvae had characteristics close to mammals. The insect species evaluated would be alternative biomodels without juridical limitations concerning animal welfare and which would moreover be cheaper (Berger 2009a).

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