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Short communication

Phagocytosis mediates specificity in the immune defence of an invertebrate, the woodlouse *Porcellio scaber* (Crustacea: Isopoda)

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ABSTRACT

Specificity and memory are the hallmarks of the adaptive immune system of vertebrates. However, phenomena of specificity upon priming of immunity have recently been demonstrated also in invertebrates, which rely exclusively on innate immune defence. It has been suggested that phagocytosis might represent a core candidate for such specificity in invertebrates. We here developed *in vitro* phagocytosis measurements for different bacteria in the woodlouse *Porcellio scaber* (Crustacea: Isopoda). After immune priming with heat-killed bacteria, hemocytes showed increased phagocytosis of a previously encountered bacterial strain compared to other bacteria. These data support the role of phagocytosis in invertebrate immunological specificity and suggest a high degree of specificity that even enables to differentiate between strains of the same bacterial species.

1. Introduction

Parasites do not only reduce host fitness but also impose strong selection pressures on their hosts by continuously evolving new ways to evade host defence. This may result in an arms-race between hosts and parasites [1,2] and the evolution of efficient immune systems [3,4]. The pressure of parasites is likely to be quite similar across host taxa. Microbial invaders, with short generation times permanently produce new genotype variants, which need to be recognized and controlled by immune defences capable of specific defences and adaptation. Already four decades ago this question motivated research on specific immune priming in invertebrates. Those studies suggested some degree of specificity in invertebrate immunity [5,6], but due to incomplete experimental designs that lacked reciprocal primary and secondary exposure, and due to the lack of mechanistic knowledge, the topic remained controversial [7–11].

Recently, well-controlled studies demonstrated not only a high degree of specific responses towards different infections [12] but also a high degree of specific immune priming in invertebrate immunity [13–15], analogous to the vertebrate specific immune memory [9,11,16,17]. Phagocytic blood cells are central to innate defence in both invertebrates and vertebrates, where they are also critical for linking innate to adaptive immunity [18–24]. Moreover, phagocytosis seems relevant for one of the most promising candidates for specific immunity in insects and crustaceans, the alternatively spliced Down syndrome cell adhesion molecule (Dscam) [18,19]. We therefore here focused on phagocytosis as a potential mechanistic underpinning of immunological specificity in invertebrates.

Enhanced phagocytosis resulting from prior exposure to endotoxin [25] or live pathogenic bacteria [26,27] has previously been demonstrated in invertebrates. However, these studies lacked a reciprocal exposure to another pathogen, such that a general increase of immunity after bacterial priming could not be distinguished from a specific enhancement of immune response against the respective pathogen. Only the latter would allow concluding that increased phagocytosis upon immune priming was a pathogen-specific rather than a general effect.

Indirect evidence that phagocytosis is involved in mediating the specificity in immune priming comes from *Drosophila melanogaster* and the bacterium *Streptococcus pneumoniae* [28]. When phagocytosis was blocked by injection of polysterene beads prior to infection, the protective effect of immune priming disappeared and naïve and primed flies died at the same rate [28].

However, a direct demonstration that phagocytic activity is increased in a pathogen-specific manner after priming would

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require a fully reciprocal design, using homologous and heterologous combinations of bacteria for priming and later challenge. For this we developed *in vitro* phagocytosis assays for an invertebrate, the woodlouse *Porcellio scaber* (Crustacea: Isopoda). *P. scaber* was chosen as a representative of another phylogenetic line of arthropods and because of its large size allowing several immune measurements per animal. For priming, we exposed the animals to a variety of heat-killed bacteria and then compared *in vitro* phagocytosis against the previously encountered and all other bacteria. Moreover, to get a first estimate of the degree of specificity, i.e. the level to which phagocytes are able to differentiate among pathogens, we used bacteria of different degrees of similarity (i.e., different Gram types, species and strains).

2. Materials and methods

Two months before the start of the experiment (spring 2008) 300 adult woodlice of the species *P. scaber* were collected in a forest in Münster-Gelmer (North Rhine-Westphalia, Germany). To standardize living conditions woodlice were kept for 2 months singly in Petri dishes half filled with gypsum to keep humidity constant after adding water. Twice a week the animals were fed with artificial food *ad libitum* [29] and water was added.

For the experiment 150 animals (30 per treatment) were selected at random for priming with heat-killed bacteria. For this, bacteria of the species *Bacillus thuringiensis* strain 1 (Bt1; DSM no. 2046), *B. thuringiensis* strain 2 (Bt2; DSM no. 6073) and *Escherichia coli* (Ec; DSM no. 498) were grown in an over-night culture, centrifuged (2000 g) and the culture medium was exchanged with sterile Ringer (saline solution). Thereafter the bacteria were heat-killed (heat-block for 30 min at 90 °C). Bacteria were counted and adjusted to a concentration of 10^{10} bacteria cells/ml (for details see [30]).

For priming, an insect needle (0.025 mm diameter) was dipped into the bacteria solution and the woodlice were pricked on the dorsal side between the second last and the last segment. As controls, animals were pricked with Ringer solution without bacteria ($n = 30$) or left naïve ($n = 30$). After this, the animals were put back into the Petri dishes, survival was checked regularly and food and water were renewed twice a week.

Two weeks later, from each surviving animal (survivorship per group: Bt 1: 18, Bt 2: 18, Ec: 18, Rin: 21, Naïve: 27; total: 102 animals) 3 μl of fresh hemolymph were collected by pricking the animals with an insect needle and collecting the outflowing droplet of hemolymph in three 1 μl capillaries coated with PTU (capillary was dipped into a saturated solution of phenylthiourea in methanol, and only used after evaporation) and cooled on ice. 1 μl of hemolymph was added to each of three wells of a Lab-Tek Chamber slide filled with 250 μl of Grace Insect Medium, to obtain three phagocytosis measures per animal, one per bacterium.

The *in vitro* phagocytosis quenching assay was performed as described in detail in [31] with the following changes. After addition of hemolymph the Lab-Tek Chambers were kept for 15 min on ice and were then put into a wet chamber at room temperature for half an hour. During this time, hemocytes could attach to the slide. Thereafter, the phagocytic activity was quantified by offering fluorescein-isothiocyanate (FITC)-labeled bacteria, labeling was done according to standard protocols. One of the three different heat-killed and fluorescently labeled bacteria species (*B. thuringiensis* strain 1, *B. thuringiensis* strain 2, *E. coli*) was added per well, resulting in all three bacteria per animal, at a concentration of 10^7 bacteria/well in 50 μl Grace. For incubation, the slides were put back into the wet chamber. After 2 h the slides were washed with Grace several times, trypane blue was added to quench the fluorescent bacteria outside of the hemocytes (i.e. non-phagocytosed bacteria). After 15 min, trypane blue was washed away with Grace until the slides were almost not blue anymore. DAPI was added, most liquid and the upper part of the slide was taken away, glycerin gelatine was added and the slide was mounted with a coverslip.

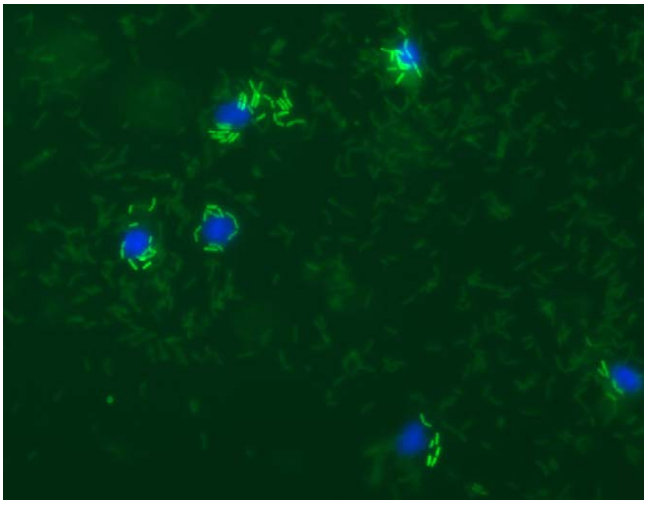

Fig. 1. Hemocytes of *P. scaber* after *in vitro* phagocytosis of *B. thuringiensis*. The nuclei of the hemocytes were labeled with the nucleic acid dye DAPI (blue color), bacteria were labeled with FITC. Only internalized bacteria retain their fluorescence (bright green) after quenching with trypan blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

During the next days the phagocytic activity was quantified by counting the number of phagocyting and non-phagocyting hemocytes on an epifluorescent microscope at 400× magnification (Fig. 1). Per well, 10 fields of view in a fixed order were quantified. This represented on average 76 (SEM 3.033) hemocytes or about 5% of all incubated hemocytes. As our measure of the phagocytic activity, the number of phagocyting hemocytes was divided by the total number of hemocytes. All animals were sacrificed and the sex was determined using a stereo microscope (30× magnification).

To determine whether survival was affected by the priming treatment with heat-killed bacteria a one-way Proportional Hazard analysis was performed. In the main statistical analysis consisting of a three-way ANOVA, we investigated whether the fixed effects priming, challenge and sex had an impact on phagocytic activity. To reach a normal distribution of the data, the phagocytic activity (hemocytes phagocyting/total number of hemocytes) was arcsin(sqrt) transformed. For a detailed analysis the significant main effects were further investigated in a post hoc analysis (Tukey's HSD test). All analyses were performed in JMP 6 (SAS Institute Inc.)

3. Results

3.1. Survival between priming and challenge

There were no significant differences in survival (until 14 days after challenge) in the effect of the three bacteria species used for priming. There was a non-significant trend for naïve animals to survive better than all pricked counterparts (SOM Table 1). Only Ringer and Naïve animals did have non-overlapping confidence intervals suggesting a significant difference.

3.2. Phagocytic activity

Immune priming had a significant effect on phagocytic activity (proportion of phagocyting cells; Figs. 1 and 2, Table 1 and SOM