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Losing the battle against fungal infection: Suppression of termite immune defenses during mycosis

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ABSTRACT

The dampwood termite, *Zootermopsis angusticollis* is known to generate humoral immune responses to the entomopathogenic fungus *Metarhizium anisopliae*. However, little is known about how the termite's cellular immune system reacts to fungal infection. To test the effect of conidia exposure on cellular immunity, we quantified the number and types of hemocytes in the hemolymph of naïve nymphs and compared their circulating counts with those of nestmates exposed to 0, 2×10^3, 2×10^6 or 2×10^8 conidia/ml doses. These termites were then bled and their hemocytes counted on days 1, 2, 3, 4, 7 post-exposure. Our results show, first, that naïve *Z. angusticollis* nymphs have three different blood cell types tentatively identified as granular hemocytes, prohemocytes and plasmatocytes. In these individuals, plasmatocytes were on average 13.5 and 3.3 times more numerous than granular hemocytes and prohemocytes, respectively. Second, a full factorial general linear analysis indicated that hemocyte type, time elapsed since conidia exposure and conidia dosage as well as all their interactions explained 43% of the variability in hemocyte density. The numbers of prohemocytes and particularly plasmatocytes, but not granular hemocytes, appear to be affected by the progression of disease. The decline in hemocyte numbers coincided with the appearance of hyphal bodies and the onset of "sluggish" termite behavior that culminated in the insect's death. Hemocyte counts of infected males and females were affected to the same extent. Hence, *M. anisopliae* overtakes the cellular immune responses of *Z. angusticollis* mainly by destroying the host's most abundant hemocyte types.

1. Introduction

In insects, several immune parameters have been used to assess immunocompetence, including lytic activity of antimicrobial peptides, rates of encapsulation against parasites or inert nylon implants, the activity of phenoloxidase and the amount of melanin and their role in encapsulation, as well as hemocyte quantification (reviewed by Lawniczak et al., 2007; Cotter et al., 2004 and references therein). In general, all these immune parameters are triggered by the highly conserved pathogen-associated molecular patterns (PAMPS, such as glycoproteins, lipopolisacharides, β-glucans, peptidoglycans), which are located in surfaces of pathogens and/or parasites (Vilcinskas and Göetz, 1999; Brown and Gordon, 2005, Schmid-Hempel, 2005; Lawniczak et al., 2007). These immunological responses, including the primed immunity in *Drosophila*, are driven by hemocytes (Lavine and Strand, 2002; Pham et al., 2007). Most studies measuring the different aspects of immune function have been carried out in solitary insects within the orders Diptera, Lepidoptera, Hemiptera, Dictyoptera, Orthoptera, Coleoptera and Mecoptera (Arnold, 1979; reviewed by Lawniczak et al., 2007, and references therein). However, termites and other social insects are excellent test organisms to study immune responses, given that they exploit microbially rich environments and engage in frequent close-range social interactions with nestmates, conditions that could exacerbate the risk of disease transmission (reviewed in Rosengaus et al., 2011).

Within the Isoptera, the dampwood termite, *Zootermopsis angusticollis*, has been used extensively to study behavioral and biochemical adaptations that reduce the likelihood of invasion by pathogens and parasites (reviewed in Rosengaus et al., 2011). Several of these adaptations appear to be socially mediated (Traniello et al., 2002). But once the pathogen/parasite crosses the cuticle and invades the insect's hemocoel, other internal physiological responses are recruited. Previous *in vivo* experiments show that immunologically primed *Z. angusticollis* are protected for up to seven days following an otherwise lethal challenge with the active bacterium *Pseudomonas aeruginosa* or a lethal dose of *Metarhizium anisopliae* fungal conidia (Rosengaus et al., 1999). Termites respond at the humoral level by upregulating the production of constitutive proteins and producing novel proteins with antifungal activity (Rosengaus et al., 2007; Bulmer et al., 2009).

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Unfortunately, very little information exists on the cellular component of their immune system (Calleri et al., 2007) and nothing is known about the dynamics between their cellular immunity and the progression of fungal infection.

The aims of this study were twofold. First, we estimated the baseline cellular immunocompetence of *Z. angusticollis* by identifying and quantifying the number of circulating hemocytes of naïve termites. Typically, it is assumed that a higher density of hemocytes reflects a better ability to mount an efficient immune response (Lawniczak et al., 2007): a higher number of circulating blood cells can readily detect components of the fungal cell wall (i.e. β-1,3 glucans) which in turn induce phagocytosis and the upregulation of antimicrobial peptides (Brown and Gordon, 2005). Second, we studied the impact that *M. anisopliae* infection has on the relative proportions of circulating hemocyte types. If the termite host controls fungal infection through cellular immunity, we predict an increase in hemocytopoiesis activity (as defined by Jones, 1979) and/or rates of hemocyte differentiation. Alternatively, if the fungus has coevolved adaptations to circumvent the host's immune responses, then the successful invasion of *M. anisopliae* should negatively impact the host's cellular immune responses.

2. Material and methods

2.1. Termite collection and colony maintenance

Mature colonies of *Z. angusticollis* were collected from Memorial Park, San Mateo County, California. Termites were fed decayed birch *ad libitum* and maintained at 25 °C inside covered plastic boxes (50 cm × 30 cm × 20 cm) lined with moist paper towels.

2.2. Classification and quantification of hemocytes in Z. angusticollis

Using a basic staining technique and based on the detailed morphological descriptions by Jones (1979), Gupta (1979), Arnold (1979), Gillespie et al. (1997), Ribeiro and Brehélin (2006) and Manfredini et al. (2007) we tentatively identified three morphologically distinct hemocyte types in *Z. angusticollis*: Type I (granular hemocytes), Type II (prohemocytes) and Type III (plasmatocytes). Although the nomenclature and classification of insect hemocytes have important limitations (Jones, 1979; Gupta, 1979; Arnold, 1979; Ribeiro and Brehélin, 2006; Manfredini et al., 2007), the consistent use of the following identification criteria was useful in distinguishing the three hemocyte types (Fig. S1): granular hemocytes contained dense granules, had a small nucleus and roundish shape; prohemocytes were characterized by their round, flat shape and typical large nuclear:cytoplasmic ratio; plasmatocytes were spherical cells with a centralized nucleus and a cytoplasm that lacked granules but contained clear vacuoles.

To establish a baseline relative proportion of each of these hemocyte types, 40 naive nymphs (13 females and 27 males) were bled. Prior to bleeding, termites were cold immobilized and their abdomen swabbed with 70% alcohol. Once the alcohol evaporated, a few crystals of phenylthiourea were placed on the abdominal cuticle to prevent coagulation and melanization of the 3 μl of hemolymph drawn from each insect. The sample was collected into a pipette tip already containing 1 μl of cold (4 °C) Burnes Tracey saline (Ludwig et al., 1957) and then transferred into a cold microcentrifuge tube with 7 μl of trypan blue stain to better visualize the hemocytes. The stain/hemolymph mixture remained on ice for 2 min. Subsequently, after gentle shaking, 9 μl of this sample was transferred onto a hemocytometer and hemocyte types were identified and counted.

2.3. Effect of fungal disease on hemocyte counts

The *M. anisopliae* sample originated from the American Type Culture Collection (batch 93-09, media 325, ATCC #90448) and fresh viable cultures are continuously maintained in our lab by isolating conidia from infected termite cadavers. To determine the impact of the entomopathogenic fungus *M. anisopliae*, a common natural pathogen of termites, on the density of hemocytes during the progression of fungal disease, we used 217 additional nymphs (87 females and 130 males) from the same colony as the naïve individuals (Section 2.2). Prior to exposure to fungal conidia, each individual was sexed and weighed. Subsequently, termites were exposed to a 3 μl droplet of a control Tween 80 suspension medium lacking fungal conidia ($n = 95$), or a 2×10^3 ($n = 35$), 2×10^6 ($n = 47$), or 2×10^8 ($n = 40$) conidia/ml suspension following the protocols described by Traniello et al. (2002). The conidia suspension was highly viable, with an average (±S.D.) percent germination = 91% ± 0.005 ($n = 30$ fields of vision). After exposure, termites were kept undisturbed in sterile plastic Petri dishes lined with filter paper (Whatman # 1) moistened with 1 ml sterile water and bled on days 1, 2, 3, 4, 7 post exposure. No termite was bled more than once.

2.4. Statistical analyses

After controlling for the effects of gender, termite mass, time since conidia exposure, conidia dose and hemocyte type, diagnostic normality tests on the residuals of the main model indicated that hemocyte counts were not normally distributed [skewness = 2.3 ± 0.1 (S.E.), kurtosis = 6.6 ± 0.3 (S.E)]. Thus, the same model was run with log-transformed hemocyte density data, and this time, the residuals of hemocyte counts were roughly normalized [skewness = 0.3 ± 0.1 (S.E.), kurtosis = 0.4 ± 0.3 (S.E.)]. In addition, to test to for the specific effects of conidia dose and time elapsed since conidia exposure on the log-transformed hemocyte density, General Linear Models (GLM; SPSS) were generated after controlling for effect of gender, mass, and hemocyte type. All models tested the independent contribution of each variable on hemocyte density as well as all potential interactions between and among the variables.

3. Results

3.1. Quantification of hemocyte types in un-exposed (naïve) Z. angusticollis

Naïve nymphs ($n = 40$) had on average ± S.E. a total hemocyte density of $1.7 \times 10^6 \pm 1.1 \times 10^5$ cells/ml of hemolymph (range 1.2×10^5–2.8×10^6 cells/ml). These unexposed insects differed significantly in the density of each of their hemocyte types (average granulocytes ± S.E. = $5.7 \times 10^4 \pm 1.3 \times 10^4$; average prohemocytes ± S.E. = $2.3 \times 10^5 \pm 2.7 \times 10^4$; average plasmatocytes = $7.7 \times 10^5 \pm 9.8 \times 10^4$, $F = 38.4$, df = 2, $p \leq 0.0001$, ANOVA following log-transformation). Thus, plasmatocytes were 3.3 and 13.5 times more numerous than prohemocytes and granular hemocytes, respectively. Neither gender ($F = 0.6$, df = 1, $p = 0.4$), mass ($F = 1.1$, df = 37, $p = 0.3$) nor time away from their colony ($F = 0.1$, df = 2, $p = 0.8$) significantly influenced the proportions of each hemocyte type in healthy unexposed insects (GLM).

3.2. Quantification of total hemocyte density during mycosis

We determine the influence that gender, mass, time elapsed since conidia exposure and conidia dosage had on total hemocyte counts. Our results indicated that total hemocyte count differ between experimental termites treated with 0, 2×10^3, 2×10^6 or