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Transcriptome analysis of *Anopheles stephensi–Plasmodium berghei* interactions

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Abstract

Simultaneous microarray-based transcription analysis of 4987 *Anopheles stephensi* midgut and *Plasmodium berghei* infection stage specific cDNAs was done at seven successive time points: 6, 20 and 40 h, and 4, 8, 14 and 20 days after ingestion of malaria infected blood. The study reveals the molecular components of several *Anopheles* processes relating to blood digestion, midgut expansion and response to *Plasmodium*-infected blood such as digestive enzymes, transporters, cytoskeletal and structural components and stress and immune responsive factors. In parallel, the analysis provide detailed expression patterns of *Plasmodium* genes encoding essential developmental and metabolic factors and proteins implicated in interaction with the mosquito vector and vertebrate host such as kinases, transcription and translational factors, cytoskeletal components and a variety of surface proteins, some of which are potent vaccine targets. Temporal correlation between transcription profiles of both organisms identifies putative gene clusters of interacting processes, such as *Plasmodium* invasion of the midgut epithelium, *Anopheles* immune responses to *Plasmodium* infection, and apoptosis and expulsion of invaded midgut cells from the epithelium. Intriguing transcription patterns for highly variable *Plasmodium* surface antigens may indicate parasite strategies to avoid recognition by the mosquito's immune surveillance system.

Keywords: *Plasmodium*; *Anopheles*; Gene expression

1. Introduction

Plasmodium, the causative agent of malaria, exploits the female mosquito's need for blood to spread among human hosts. Sets of different digestive enzymes, transporters, structural components, metabolic enzymes and developmental factors are required by the mosquito vector to process the blood and produce eggs. The presence of *Plasmodium* poses additional challenges for the mosquito and triggers transcriptional programs responsible for immune and stress response, apoptosis, tissue healing and other physiological systems that are affected by infected blood components [1,2]. *Plasmodium*

Abbreviations: CSP, circumsporozoite protein; CTRP, circumsporozoite- and TRAP-related protein; EST, expressed sequence tag; NOS, nitric oxide synthase; Nramp, natural resistance-associated macrophage protein; RT-PCR, reverse transcription-polymerase chain reaction; TRAP, thrombospondin-related adhesive protein; WARP, von Willebrand factor A domain-related protein

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undergoes a complex life cycle in the mosquito and the temporal accuracy of its gene regulatory program is crucial to avoid exposure of the sensitive parasite stages to the hostile environment of the midgut lumen and the mosquito's immune responses in the epithelium and hemocoel [1].

Malaria development in the mosquito is initiated by gametocyte activation within the first 30 min of blood ingestion, followed by fertilization that leads to the formation of a zygote at approximately 2 h. The zygote transforms into a motile ookinete that traverses the peritrophic matrix using a chitinase that may be activated by a mosquito digestive protease. After entering the ectoperitrophic space approximately 15 h after ingestion, the parasite attaches to, invades and traverses the midgut epithelium. Invasion of the midgut by the rodent malaria parasite *Plasmodium berghei* peaks at around 30 h after infected blood ingestion and proceeds up to 2 days. Large numbers of parasites are killed prior to and during invasion of the midgut epithelium. Invaded midgut cells will undergo apoptosis and finally be expelled from the epithelium. On the basal side the ookinete develops, within a period of approximately 12–20 days, into a mature oocyst that produces thousands of sporozoites [1,3]. Upon the rupturing of oocysts, which is asynchronous and can take several days, sporozoites are dispersed throughout the hemocoel from where they will invade the salivary glands prior to injection into a new host.

Our knowledge of *Anopheles* responses to malaria infected blood and *Plasmodium's* development in the mosquito has experienced a remarkable expansion in the past decade mostly through individual gene analyses for both organisms. Recent gene discovery projects based on subtraction cDNA libraries that were enriched with both *Anopheles stephensi* and *P. berghei* infection stage specific genes, identified numerous new transcripts that are crucial for the interactions between the two organisms [4,5]. However, subtractive hybridization approaches cannot provide accurate temporal transcription patterns of genes across several consecutive time-points, and expression stage specificities of selected genes must therefore be determined by other methods.

To extend our understanding of *Anopheles–Plasmodium* interactions, we have simultaneously assayed gene expression of *A. stephensi* midguts and *P. berghei* at seven time-points after mosquitoes fed on a *P. berghei* infected mouse. Analyses were done with microarrays made from EST clones of subtraction libraries enriched for *Plasmodium* genes expressed in *Anopheles*, and *Anopheles* genes induced by *Plasmodium* infected blood. The analyses reveal processes of *Plasmodium* development, blood digestion and the mosquito's response to infection.

2. Material and methods

2.1. Mosquito rearing and infections

The *A. stephensi* SD500 strain was raised at 28 °C, 75% humidity, under a 12 h light/dark cycle, and maintained on a 10% sucrose solution during adult stages. Female mosquitoes were blood-fed on anaesthetised BALB/c mice. For malaria infections 4-day-old female mosquitoes were fed on anaesthetised BALB/c mice which had been infected with *P. berghei* 4 days previously, and were assayed for high levels of parasitemia and the presence of microgametocytes capable of exflagellation. The mosquitoes were maintained thereafter at 19 °C prior to dissection and extraction of midgut RNAs.

2.2. Dissections and RNA extraction

Midguts were dissected on ice in PBS (0.6 mM $MgCl_2$, 4 mM KCl, 1.8 mM $NaHCO_3$, 150 mM NaCl, 25 mM HEPES, 1.7 mM $CaCl_2$, pH 7) and were immediately frozen on dry ice. Total RNA was prepared from dissected tissues and intact animals using the RNeasy kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

2.3. DNA microarray construction

The EST clones that were used for microarray probe preparation originated from four previously described subtraction libraries that represented the transcriptomes of the ookinete stage *P. berghei* (ookinete library: 1–12 h) and the early infected stage (early library: 24–52 h after ingestion of infected blood), intermediate oocyst stage (middle infected midgut library: 4, 6 and 8 days after ingestion of infected blood) and late oocyst stage (infected midgut library: 10, 12 and 14 days after ingestion of infected blood) infected *A. stephensi* midgut and *P. berghei* transcripts [4,5]. Subtraction of cDNAs was done using the Clonetech PCR select subtraction kit (cat. # K1804-1) and the subtracted PCR amplified cDNA fragments were cloned in a pGEMT-easy (Promega) vector prior to transformation into DH5α *Escherichia coli*. The ookinete subtraction library was constructed by subtracting cDNAs from four ookinete stages (6, 12, 18 and 24 h) against blood stage *P. berghei* cDNAs. The early subtraction library was produced by subtracting cDNA from infected midguts at 24, 36 and 52 h after ingestion against cDNAs of blood stage *P. berghei* and cDNAs of non-infected blood fed guts at 24 h after ingestion. The middle subtraction library (representing cDNAs from 4, 6 and 8 days after ingesting infected blood) was subtracted against blood stage *P. berghei* cDNA and cDNAs of non-fed guts, and 24 and 48 h, and 4 days non-infected blood fed guts. To ensure high degree of stage specificity, the middle library had also been subtracted against cDNAs of 24 and 48 h infected guts. The late subtraction library (representing cDNAs from 10, 12 and 14 days after ingesting infected blood) was subtracted against the same cDNAs the middle subtraction library and in addition against cDNAs of infected guts at 4, 6 and 8 days after ingestion. Detailed description of these libraries is provided in previously published work [4,5]. Probes for spotting were amplified from 4987 EST clone bacterial cultures through a two-step PCR amplification with amino-modified T3 and