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A haemocyte tropism for an arbovirus

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Horizontally transmitted mosquito-borne viruses enter the midgut with a blood meal then disseminate to infect the salivary glands. En route to the salivary glands, these viruses encounter the plasma (haemolymph) and blood cells (haemocytes). Haemocytes respond to a variety of micro-organisms, but their role in virus replication and dissemination has not been described. To look for a potential haemocyte tropism for an arbovirus, a Sindbis virus was injected intrathoracically into four species of mosquito. Virus infects haemocytes as early as 6 h post injection (h p.i.) and infection was evident in these cells for as long as 4 days p.i. More than 90% of haemocytes were infected, most often the phagocytic granulocytes. Virus titres in the haemolymph increased from 24 h p.i. through 60 h p.i. Similar results were found when *Aedes aegypti* mosquitoes were injected with orally infectious Sindbis. These data prove that an arbovirus infects, and replicates in, haemocytes.

Mosquito-borne arboviruses initiate infection in the midgut of the mosquito following ingestion of a viraemic blood meal. As the infection progresses, the virus replicates and disseminates from the midgut then invades and replicates in the salivary glands. There is little information regarding dissemination of virus from the midgut to the salivary glands. It is postulated that the virus requires 1) a means to amplify after its escape from midgut epithelial cells, so that it can infect the salivary glands efficiently (Girard *et al.*, 2004; Hardy *et al.*, 1983), and 2) a vehicle to ensure dissemination from the midgut to the salivary glands (Romoser *et al.*, 2004).

In lepidopteran larvae infected with baculoviruses, virus entry into midgut epithelial cells is followed by infection of the tracheoles, the oxygen transporting tissue in insects. In several lepidopteran species, insect blood cells, called haemocytes, facilitate amplification of baculoviruses as well as virus dissemination from tracheoles to secondary organs (Engelhard *et al.*, 1994; Keddie *et al.*, 1989). Resistance to baculovirus infection in some species is attributed to haemocytes avoiding infection, and also exhibiting apoptosis-like behaviour (Clarke & Clem, 2002; Feng *et al.*, 2007; Trudeau *et al.*, 2001).

In the context of mosquito-borne viruses, the route of virus dissemination is not well understood. Work with vector–virus systems including *Aedes albopictus*–Sindbis virus (SINV), *Aedes aegypti*–Dengue virus (DENV), *Ochlerotatus taeniorhynchus*–Venezuelan equine encephalitis virus and *Culex pipiens pipiens*–Rift Valley fever virus indicates the presence of viral antigen in tracheoles, which might shuttle the virus across the midgut basement membrane to initiate dissemination (Bowers *et al.*, 2003; Romoser *et al.*, 2004; Salazar *et al.*, 2007). Midgut muscle tissue is proposed also as a means of amplification and dissemination for West Nile virus (WNV) (Girard *et al.*, 2004).

Whether via the tracheoles or muscle tissue, mosquito-borne arboviruses inevitably encounter the haemocoel environment and haemocytes en route to the salivary glands. In response to pathogens, mosquito haemocytes phagocytose bacteria, yeast and *Plasmodium* parasites; mediate melanization of some nematode parasites, yeast and bacteria; and are involved in cell signalling events for the production of antimicrobial peptides together with the fat body (Bartholomay *et al.*, 2004; Hernandez-Martinez *et al.*, 2002; Hillyer *et al.*, 2003a, b, 2004; Infanger *et al.*, 2004; Lowenberger, 2001). The role of mosquito haemocytes in arbovirus replication, dissemination and antiviral responses is unknown. Infection of mosquito haemocytes with an arbovirus has been noted, but not characterized to date (Foy *et al.*, 2004; Salazar *et al.*, 2007; Sriurairatna & Bhamarapravati, 1977), which is not surprising, because these cells are few and difficult to collect (Castillo *et al.*, 2006; Hillyer & Christensen, 2002).

Because haemocytes are critical to the outcome of baculovirus infections in lepidopteran larvae, and mosquito-borne arboviruses must traverse the haemocoel to get to the salivary glands, and haemocytes in the haemocoel respond to a variety of pathogens, we hypothesized that haemocytes respond to virus present in mosquito haemolymph. In order to explore the potential role of haemocytes in the amplification and dissemination, or mitigation, of arbovirus infection, we observed haemocytes after infecting mosquitoes with SINV (family *Togaviridae*, genus *Alphavirus*) transducing viruses designed to drive production of green fluorescent protein (GFP) (see Pierro *et al.*, 2003).

Mosquitoes were infected with SINV TE/5'2J/GFP or MRE16-eGFP via intrathoracic injection. Intrathoracic injection is a standard procedure for delivering a consistent dose of virus to each experimental mosquito (see Bowers *et al.*, 1995, 2003). Also, for comparative purposes, intrathoracic injection was the only method by which mosquitoes could be infected with both TE/5'2J/GFP and MRE16-eGFP because TE/5'2J/GFP is not infectious *per os* (Foy *et al.*, 2004; Pierro *et al.*, 2003). However, it should be noted that needle injection elicits a rapid cellular and humoral immune response in mosquitoes (Lai *et al.*, 2001) and is not a natural route of arbovirus infection. Both TE/5'2J/GFP and MRE16-eGFP were produced using standard techniques (see Olson *et al.*, 2000) and a volume of 0.5 μl of 1×10^8 p.f.u. ml^{-1} was injected per mosquito in all experiments. Haemolymph was collected by perfusion onto microscope slides.

Initial experiments were done with *Ae. aegypti* (Liverpool strain), a primary vector of Yellow fever and DENV viruses. Haemocyte infection was observed in three separate experiments at 6, 12, 18, 24, 36, 48, 60, 72, 84 and 96 h post injection (h p.i.) using fluorescence and phase microscopy. Haemocytes from TE/5'2J/GFP infected mosquitoes exhibit GFP expression as early as 6 h p.i. and for 4 days p.i (Fig. 1). Although oenocytoids sometimes appear infected, granulocytes are the haemocyte type most often infected with virus. Granulocytes are the most abundant circulating haemocyte type; these phagocytic cells attach to glass surfaces and are easily distinguished by pseudopodia (Castillo *et al.*, 2006; Hillyer & Christensen, 2002). At 48 h p.i. and at every time point thereafter, virus-infected cells exhibit bulbous extensions of the cytoplasm and plasma membrane. At 84 h p.i., the intensity of GFP fluorescence and number of infected granulocytes begins to decline. This does not appear to be a result of cell death, because total haemocyte numbers do not decrease.

Using one cohort of mosquitoes, infected haemocytes were observed and counted based on the presence or absence of GFP fluorescence. The proportion of infected cells throughout the time-course is as follows: 45% (6 h p.i.), 88% (12 h p.i.), 96% (18 h p.i.),

100% (24 h p.i.), 89% (36 h p.i.), 87% (48 h p.i.), 83% (60 h p.i.), 86% (72 h p.i.), 75% (84 h p.i.) and 70% (96 h p.i.).

In keeping with previous reports on TE/5'2J/GFP where virus was first observed 3 days p.i. (Pierro *et al.*, 2003, 2007), virus is first observed in head squashes at 60 h p.i. in 87.5% (7/8) of *Ae. aegypti*. At all later time points (72, 84 and 96 h p.i.), 100% (10/10, 6/6 and 7/7 mosquitoes, respectively) of head tissues are virus infected.

GFP expression and accumulation is a valid measure of SINV-GFP replication (Foy *et al.*, 2004; Olson *et al.*, 2000; Pierro *et al.*, 2003). However, to further confirm that virus replication takes place in haemocytes, the titre of TE/5'2J/GFP in *Ae. aegypti* haemolymph was calculated using an end-point dilution assay at different time points post injection, and expressed as a tissue culture infectious dose 50% (TCID₅₀) (Fig. 2). Haemolymph from saline-injected mosquitoes was used as a negative control and did not cause cytopathic effects. Virus titres from mosquito haemolymph are lowest at 6 h p.i., and increase over time. Titres from haemolymph collected at 24, 36, 48 and 60 h p.i. are significantly higher than those at earlier (6, 12, and 18 h p.i.) or later (72, 84, and 96 h p.i.) time points. The highest titre log TCID₅₀ ml⁻¹ (9.77±0.52) is observed at 48 h p.i. in the haemolymph of infected mosquitoes.

The increase in virus titre observed in haemolymph samples is likely solely attributed to virus replication in haemocytes because 1) the tissue tropisms for this virus are limited (Pierro *et al.*, 2007), 2) infection is not evident during the observation period in the midgut, Malpighian tubules and ovaries in this (data not shown) and a previous study (Olson *et al.*, 1996), and 3) virus amplification is not evident in head tissue until 60 h p.i., 26 h after a significant increase in haemolymph virus titre occurs. Furthermore, in two previous studies in which SINV was used to silence phenol oxidase (PO), PO suppression was evident for 15 days p.i. (Shiao *et al.*, 2001; Tamang *et al.*, 2004). Because these viruses produce the gene of interest from a subgenomic promoter, antisense PO production necessitates virus replication, and because PO is a haemocyte-specific gene, virus replication had to occur in haemocytes.

To investigate whether the observed phenomenon was species specific, haemocytes from additional species of mosquitoes (*Aedes triseriatus*, *Ae. albopictus* and *Culex pipiens*) were observed after injection with TE/5'2J/GFP. Experiments were done with at least three different cohorts of mosquitoes. Haemocytes were checked for GFP expression at 24 and 48 h p.i., when the highest percentage of virus-infected haemocytes is observed in *Ae. aegypti*. In all three species, virus-infected haemocytes are evident at 24 and 48 h p.i. (Fig. 3a–l). The proportion of haemocytes infected with the virus is as follows at 24 and 48 h

p.i.: 69% and 76% (*Ae. triseriatus*), 72% and 65% (*Ae. albopictus*), 53% and 65% (*Cx. pipiens*). As seen with *Ae. aegypti*, granulocytes exhibit the most GFP fluorescence in TE/5'2J/GFP-infected *Ae. triseriatus*, *Ae. albopictus* and *Cx. pipiens*.

Cx. pipiens exhibit fewer fluorescent haemocytes, and GFP fluorescence intensity is lower than that of *Aedes* species granulocytes, but the proportion of infected haemocytes is not significantly different according to Tukey-Kramer HSD analysis (data not shown). In another study of *Culex*–SINV tissue tropisms, midguts were almost completely resistant to oral infection (Foy *et al.*, 2004).

Infection of the head tissue of *Ae. triseriatus*, *Ae. albopictus* and *Cx. pipiens* is detected at 48 h p.i. in 88.8% (8/9) of mosquitoes of all three species, earlier than was observed in the head tissue of infected *Ae. aegypti*.

SINV MRE16-eGFP was injected into the haemocoel of *Ae. aegypti* mosquitoes to investigate whether haemocytes can be infected with a virus that has the full complement of genes required to infect and disseminate from the midgut (see Foy *et al.*, 2004; Myles *et al.*, 2003). Haemocytes from *Ae. aegypti* injected with MRE16-eGFP exhibit fluorescence at 24, 48, 72 and 96 h p.i. (Fig. 3 m–p). The number of SINV MRE16-eGFP-infected haemocytes does not change significantly over time during this period. Granulocytes are the cell type most frequently infected with MRE16-eGFP.

Infection of *Ae. aegypti* head tissue with the MRE16-eGFP virus was assayed in head squashes in three separate experiments. Infection of head tissue is never observed at 24 h p.i. (0/12 mosquitoes tested). At 48 h p.i., 33% (5/15) and at 72 h p.i., 87% (13/15) of mosquitoes have evidence of GFP expression in the head. At 96 h p.i., 100% (12/12) of mosquitoes tested have infected head tissue.

Mosquito haemolymph is suspected to be critical for systemic arbovirus infection (see Foy *et al.*, 2004); however, it is generally believed that virions exist in a free state within the haemolymph. The results presented here show that haemocytes in the haemolymph can be infected with two different SINVs, in multiple species of mosquito. Moreover, our results show that this arbovirus can exploit haemocytes as a site for replication, rather than just existing free in the haemolymph. Since WNV takes 5–8 days to reach the salivary glands after dissemination from the midgut, Girard *et al.*, (2004) speculated that some arboviruses may require an amplification site outside the midgut epithelium. Furthermore, Hardy *et al.* (1983) speculated that low concentrations of virus in a viraemic blood meal would necessitate a secondary amplification site other than the midgut epithelium. Based on this work with SINV, it becomes clear that haemocytes can function in virus incubation and

replication, and so are a strong candidate site for arbovirus amplification en route to the salivary glands.

These data, together with previous studies indicating the involvement of tracheae in disseminating arboviruses (Romoser *et al.*, 2004; Salazar *et al.*, 2007), support an emerging model for dissemination of arbovirus, similar to the current model for baculovirus dissemination in lepidopteran larvae, where the virus disseminates from the midgut to the tracheoles and then into haemocytes that are critical for systemic infection (Engelhard *et al.*, 1994; Keddie *et al.*, 1989).

Since haemocytes are critical in the mosquito innate immune response, they could also serve as a barrier to arbovirus infection and dissemination. Documented barriers to infection and dissemination in a mosquito host include the midgut infection barrier, midgut escape barrier, salivary gland infection barrier (SIB) and salivary gland escape barrier (see Black *et al.*, 2002). Overcoming these barriers is critical for an arbovirus to successfully replicate and disseminate, and to be transmitted to the next vertebrate host. Hardy *et al.* (1983) speculated that haemolymph could be an important determinant of the SIB because virus titres in the haemolymph of *Cx. tarsalis* susceptible to Western equine encephalitis virus were significantly higher than in those of refractory females.

This is the first study to demonstrate that mosquito haemocytes are a cell type in which arbovirus infection and replication occurs. Further studies of haemocyte infection post exposure to an arbovirus via blood feeding are necessary. However, the implications of these initial findings are significant because, depending on the vector-virus system, these cells could be a site of virus amplification, serve as a conduit for virus dissemination, or present a barrier to further dissemination.

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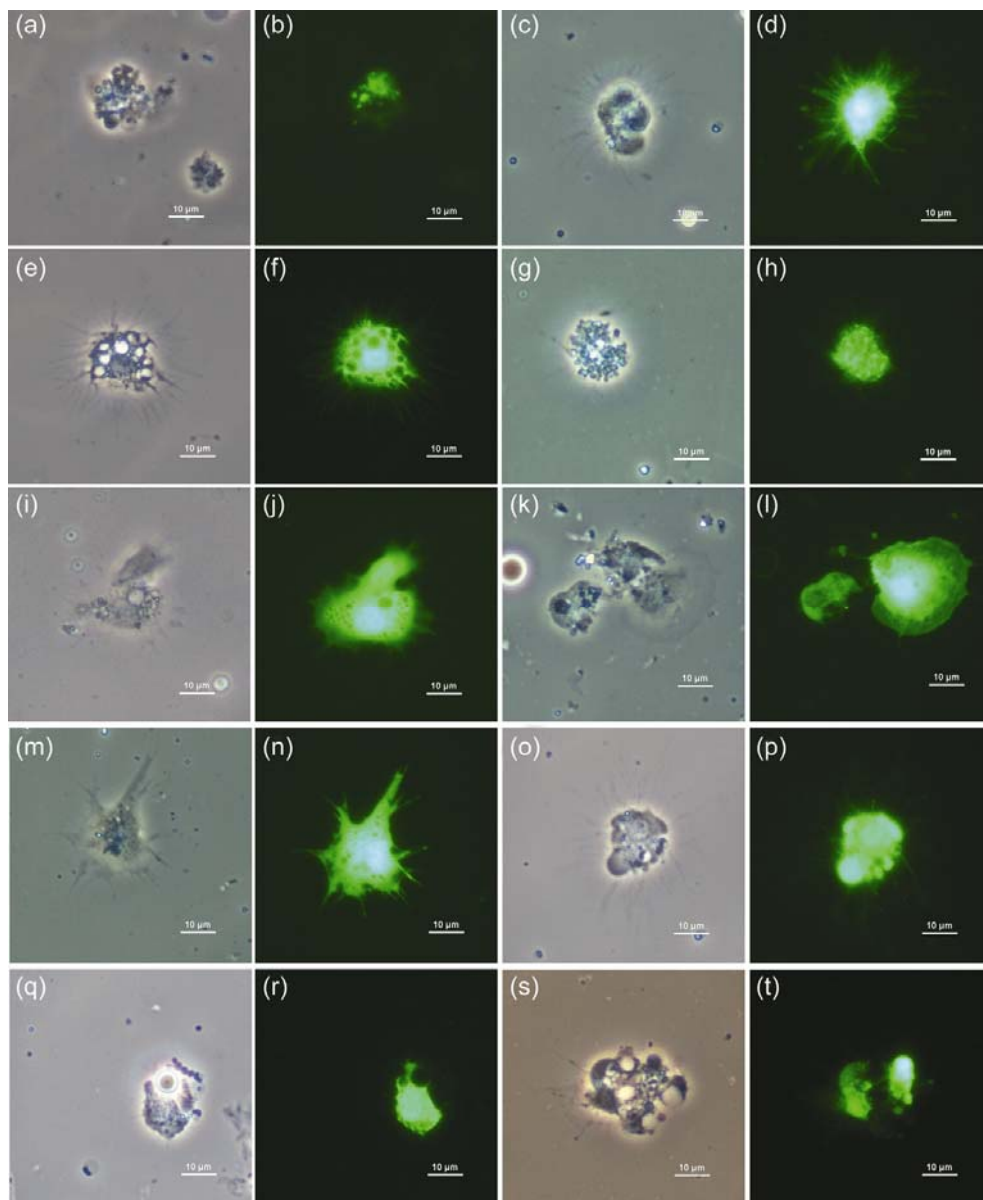


Fig. 1. *Ae. aegypti* haemocytes become infected when adult female mosquitoes are injected with SINV TE/5'2J/GFP as evidenced by production of GFP. Haemocytes were observed at 400× magnification using phase and fluorescence microscopy, shown left and right (respectively) in each pair of images. Granulocytes, characterized by the presence of pseudopodia are the haemocyte type that is most often infected with virus. Haemocytes observed at 6 (a, b), 12 (c, d), 18 (e, f), 24 (g, h), 36 (i, j), 48 (k, l), 60 (m, n), 72 (o, p), 84 (q, r) and 96 (s, t) h.p.i. are shown. Images are representative of results from three experiments. Bars, 10 μm.

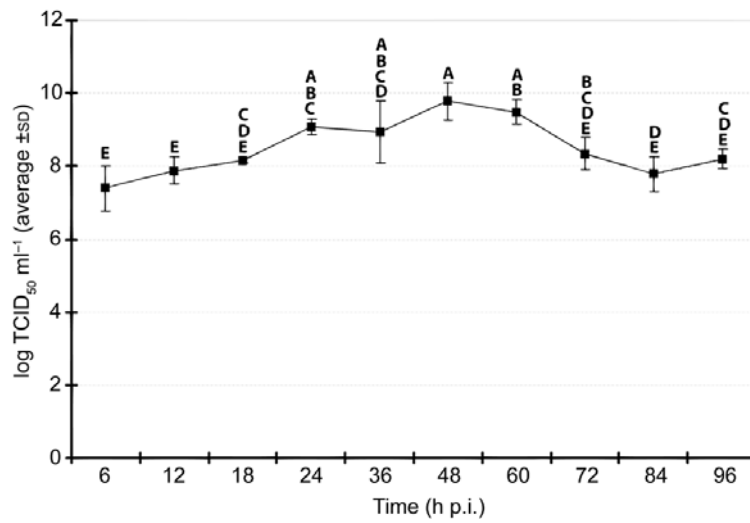


Fig. 2. Replication of SINV TE/5'2J/GFP in infected *Ae. aegypti* was measured by TCID₅₀ using Vero-76 cells, at various time points post intrathoracic injection from approximately eight drops of dilute haemolymph. Each data point is an average value from five mosquitoes. Virus replication in the haemolymph is significantly higher at 24, 36, 48 and 60 h p.i. as compared with earlier and later time points. Statistical significance was calculated using the Tukey-Kramer HSD test, displayed as letters above the error bars. Values that are not represented by the same letter are significantly different.

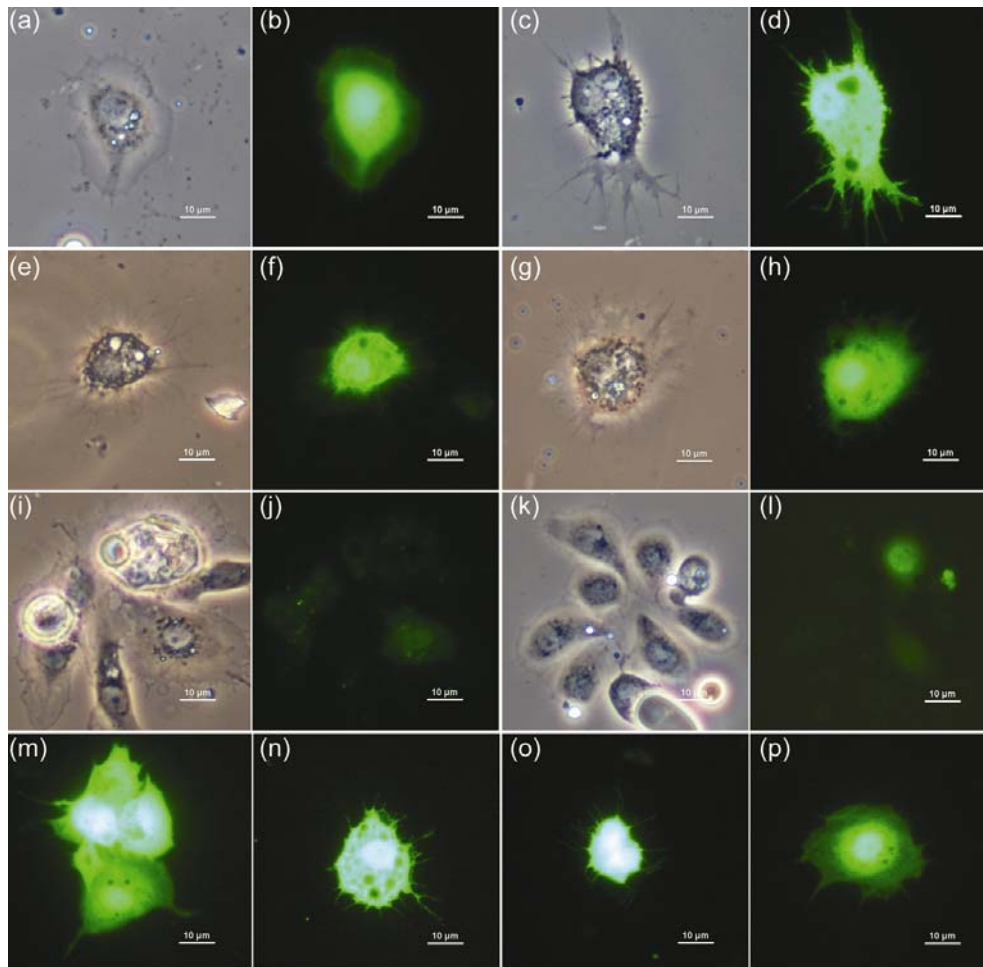


Fig. 3. Haemocyte infection is evident in multiple mosquito vector species injected with SINV TE/5'2J/GFP (top), and in *Ae. aegypti* injected with SINV MRE16-eGFP (bottom). Haemocytes were observed using phase and fluorescence microscopy shown left and right (respectively) in each pair of images. SINV TE/5'2J/GFP infection of *Ae. albopictus* haemocytes was observed at 24 (a, b) and 48 h p.i. (c, d); in *Ae. triseriatus* haemocytes at 24 (e, f) and 48 h p.i. (g, h); and in *Cx. pipiens* at 24 (i, j) and 48 h p.i. (k, l). SINV MRE16-eGFP infection of *Ae. aegypti* haemocytes was observed at 24 (m), 48 (n), 72 (o) and 96 h p.i. (p). Images i–m represent clusters of haemocytes, a–h and n–p are individual cells. Representative images from three experimental cohorts are shown. Bars, $\pm 10 \mu\text{m}$.