SUSCEPTIBILITY OF *AEDES ALBOPICTUS* TO INFECTION WITH EASTERN EQUINE ENCEPHALOMYELITIS VIRUS

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ABSTRACT. We examined susceptibility of a strain of *Aedes albopictus* from Houston, Texas to experimental infection with eastern equine encephalomyelitis (EEE) virus. After 15 days of extrinsic incubation, all *Ae. albopictus* examined by the cell culture assay and fluorescent antibody staining were infected but only 57% (4/7) of the mosquitoes that refed transmitted virus by bite. Data supported the concept of a salivary gland infection barrier to EEE virus in *Ae. albopictus* and the conclusion that virus replicates in fat body following dissemination from the midgut and prior to infection of salivary glands. Assay of adult progeny from females that fed on viremic chicks and fluorescent antibody studies of infected females failed to provide evidence that EEE virus is transmitted vertically by *Ae. albopictus.*

INTRODUCTION

Since the discovery of a breeding population of *Aedes albopictus* (Skuse) in Houston, Texas, during 1985 (Sprenger and Wuithiranyagool 1986), this species has received considerable attention from the mosquito research community and the popular press in the Americas. Besides expressing concern for its potential to become a formidable pest of humans, medical entomologists have discussed and examined the role that *Ae. albopictus* might play in North American arbovirus transmission cycles. Reviews by Shroyer (1986) and Hawley (1988) summarize the role of *Ae. albopictus* in arbovirus transmission worldwide. Published data show that the introduced strain(s) of *Ae. albopictus* can become infected with and transmit several arboviruses.

Eastern equine encephalomyelitis (EEE) virus is a highly pathogenic arbovirus that is transmitted by *Culiseta melanura* (Coquillett) among songbirds in enzootic foci along the northeast coast of the United States and south through states that border the Gulf of Mexico; there are also some inland sites of transmission (Hayes 1981, Morris 1988, Scott and Weaver 1989). In Central and South America, mosquitoes in the subgenus *Culex* (*Melanoconion*) are considered enzootic vectors of EEE virus strains that are antigenically distinct from those in North American (Casals 1964). Transmission cycles on Caribbean islands are poorly understood.

We initiated the study described herein to provide laboratory data on the susceptibility of *Ae. albopictus* to EEE virus infection. Our study is intended to be useful in assessing the risk that this species represents for transmission of EEE virus in North America. In addition, results could be compared to those from ongoing studies in our laboratory regarding EEE virus replication and dissemination in *Cs. melanura.*

MATERIALS AND METHODS

The *Ae. albopictus* used in this study were obtained from a colony of mosquitoes that were originally collected in Houston, Texas, and provided by Dr. G. B. Craig, Jr. (University of Notre Dame, Notre Dame, IN). Mosquito rearing and containment were similar to that described by Scott et al. (1984) for *Cs. melanura.* Adults were held in 30-cm cubical cages in an insectary maintained at 25°C, 80–90% RH, a photoperiod of 16 h light: 8 h dark, and a 10% sucrose solution was constantly available. Sugar was removed 24 h before mosquitoes fed on blood. Larvae were raised in plastic trays with a diet of crushed dog food and liver powder.

At 5 days postemergence, adult females were exposed for 3 h to a 1-day-old chick that had been inoculated with $10^{3.5}$ baby hamster kidney cell tissue culture 50% infective dose (BHK $TCID_{50}$) of EEE virus. Control mosquitoes were exposed to a chick that had been inoculated with diluent. The virus strain, inoculation procedure and methods for bleeding chicks are described by Scott et al. (1984). In brief, the virus strain (ME-77132) was originally isolated from *Cs. melanura* collected in Massachusetts during 1977 and had been passed once in *Cx. quinquefasciatus* Say mosquitoes and once in a C6/36 cell culture (a clone of *Ae. albopictus* cells) prior to use in this study. Birds were inoculated with 0.1 ml of a virus suspension or diluent intramuscularly in the right thigh 24 h before exposure to mosquitoes. Immediately before and after mosquito exposure, 0.2 ml of blood was taken from the jugular vein of the bird by venipuncture and mixed with 0.9 ml of diluent, centrifuged, and the supernatant was stored at −70°C until subsequent virus assay. Virus titers were determined as $TCID_{50}$ by the methods of Reed and Muench (1938) and using BHK cells grown in 96-well microtiter plates.

At 1, 2, 3, 4, 6, 8 and 15 days after engorgement, five virus exposed and 2 control mosquitoes were collected, anesthetized by chilling them on wet ice and cut in half at the junction of the abdomen and the thorax. Heads and thoraces were ground in chilled glass grinders with 0.5 ml of mosquito diluent (Scott et al. 1984). After grinding, another 0.5 ml of mosquito diluent was added, the solution was vortexed and then centrifuged at 15,000 × g for 30 sec. Specimens were stored at −70°C until assayed for virus as described above.

Abdomens were used to examine the distribution of EEE virus antigen in various organs. Organs were dissected on glass slides, fixed in acetone, stained with a direct fluorescent conjugate and examined with an epifluorescent microscope (Scott et al. 1984).

Mosquitoes that blood fed in the above experimental infection that were not cut in half were used to examine transmission competence: horizontal and vertical. To assess horizontal transmission at 8 and 15 days after engorging, 10 mosquitoes were individually exposed overnight to 1-day-old chicks. Chicks were bled 24 h later, and their bloods were assayed for virus. Seven days later, surviving chicks were again bled, and 1:10 dilutions of their sera were tested for neutralizing antibody using a constant-virus serum-dilution micro-neutralization test (Scott et al. 1984). Virus isolation or antibody detection from the blood of a recipient chick was considered evidence of virus transmission by the associated mosquito.

Vertical transmission was examined by assaying adult progeny from females that had fed on the EEE virus infected chick. Only progeny from the first egg laying cycle, immediately after feeding on the infected chick, were assayed for virus. Specimens were assayed in pools of 100 or fewer mosquitoes of the same sex in BHK cells contained in 96-well plates.

RESULTS

Figure 1 shows the replication of EEE virus over time in the heads and thoraces of *Ae. albopictus* that imbibed viremic blood. No infectious virus was detected in control mosquitoes. The EEE virus titers of blood from the donor chick before and after the mosquitoes fed on it were $10^{8.80}$ and $10^{8.55}$ BHK $TCID_{50}$ per 1.0 ml of blood. All mosquitoes that we studied fed on a single viremic chick.

Data from the cell culture assay show that the percent of mosquitoes containing detectable virus decreased during the first 2 days after blood feeding, and then began to increase during the third and fourth days of extrinsic incubation (EI). On days 8 and 15 of EI, all heads and thoraces assayed contained detectable amounts of virus (> $10^{1.75}$ $TCID_{50}$). The line on Fig. 1 connects median values only for mosquitoes with detectable titers and shows a trend of increasing titers over time.

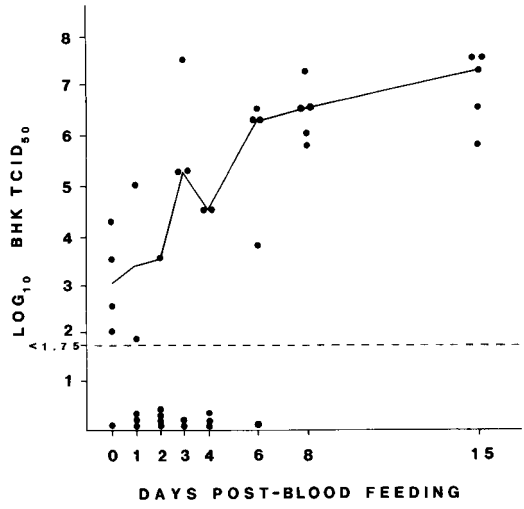

Fig. 1. Titer of heads and thoraces from *Aedes albopictus* that fed on an EEE virus infected chick. The line connects median values only for specimens with detectable titers. The titer of virus in blood from the donor chick was $10^{8.80}$ and $10^{8.55}$ BHK $TCID_{50}$ per 1.0 ml of blood before and after mosquito exposure.

A summary of EEE virus fluorescent antibody antigen detectable in abdominal organs of *Ae. albopictus* is presented in Table 1. These organs were from the same mosquitoes whose heads and thoraces were assayed for infectious virus (Fig. 1). Viral antigen was first detected 24 h after blood feeding in the posterior midgut of one mosquito. Throughout the experiment, none of the control mosquitoes contained detectable antigen. On day 2 of EI, 2 midguts contained antigen; virus had disseminated to the abdominal ganglia and fat body in one of these mosquitoes and abdominal fat body in the other. During days 3–15 all (n = 25) but one posterior midgut contained detectable EEE virus antigen. In the same time period, the next most frequently infected organ was the fat body (10/25, 40%), followed by the hindgut (8/25, 32%). The number of mosquitoes by day of EI with antigen found outside the midgut over the number with antigen detectable in the midgut (i.e., infected mosquitoes with disseminated infections) were: 0/1—day 1, 2/2—day 2, 2/5—day 3, 1/4—day 4, 3/5—day 6, 2/5—day 8 and 5/5-day 15.

During the entire study all organs other than the midgut, hindgut and fat body were infected only infrequently (6–13%), except ovarioles in which no antigen was detected. At least 30 ova-

Table 1. Distribution of eastern equine encephalomyelitis (EEE) virus fluorescent antibody antigen in organs from *Aedes albopictus* that were dissected on different days of extrinsic incubation.[a] Two control mosquitoes were dissected at each time interval; none of those specimens contained detectable antigen.

Organ	Days post-blood feeding								Total (%)
	0	1	2	3	4	6	8	15	
Anterior midgut	0/5	0	0/2	1/4	1/5	0/5	0/4	1/1	3/26 (12)
Posterior midgut	0/5	1/4	2/5	5/5	4/5	5/5	5/5	5/5	27/39 (69)
Hindgut	0/5	0/5	0/5	1/5	0/5	2/5	1/5	4/5	8/40 (20)
Rectum	0/5	0/3	0/4	1/4	0/5	1/5	0/5	2/5	4/36 (11)
Ovarioles	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/40 (0)
Lateral oviducts	0/5	0/5	0/3	0/2	0/4	1/5	0/5	1/5	2/34 (6)
Common oviduct	0/5	0	0/3	0/1	1/1	0/4	0/3	0/1	1/18 (6)
Abdominal ganglia	0/5	0/3	1/4	1/2	0/3	0/5	0/5	2/5	4/32 (13)
Dorsal vessel	0/5	0/1	0	0/4	0/3	1/5	0/4	1/4	2/26 (8)
Pericardial cells	0/5	0/1	0	1/4	0/3	1/5	0/4	0/4	2/26 (8)
Fat body	0/5	0/4	2/5	2/5	0/5	2/5	1/5	5/5	12/39 (31)
Total (%)	0/55 (0)	1/31 (3)	5/36 (14)	12/41 (29)	6/44 (14)	13/54 (24)	7/50 (14)	21/45 (47)	

[a] Mosquitoes fed on a chick circulating titers of $10^{8.80}$ and $10^{8.55}$ BHK $TCID_{50}$ of EEE virus per 1.0 ml of blood before and after the 3-h mosquito exposure.

rioles were examined in each mosquito. Patterns of characteristic specific fluorescence for *Ae. albopictus* were similar to those described previously for *Cs. melanura*; for example, in midguts and hindguts fluorescence was associated with lateral and longitudinal bands of muscle on the exterior of these organs (Scott et al. 1984). Malpighian tubules were not included in the fluorescent antibody study because of autofluorescence (Beaty and Kloter 1979, Scott et al. 1984).

On day 8 of EI, 8 of 10 *Ae. albopictus* fed on recipient chicks and 2 (25%) of those mosquitoes transmitted virus. On day 15 of EI, 7 of 10 mosquitoes refed and 4 (57%) transmitted virus.

A total of 1,657 progeny from mosquitoes that fed on the viremic chick were raised to adults and assayed for infectious virus. No virus was detected in 753 males assayed in 8 pools or in 904 females assayed in 10 pools.

DISCUSSION

Results from our experiments demonstrate that, although not an extremely efficient vector under laboratory conditions, *Ae. albopictus* can become infected with and can transmit EEE virus by bite. There was no evidence supporting vertical transmission of EEE virus by this mosquito. Viral antigen was not detected in the ovaries of females that fed on infected chicks (Table 1), and none of the progeny from those same females or their cohorts contained detectable infectious virus.

These observations of oral but not vertical transmission are not surprising. Many species of mosquitoes are susceptible to oral infection with EEE virus (Chamberlain et al. 1954a, 1954b; Chamberlain and Sudia 1955, Chamberlain 1958, Chamberlain and Sudia 1961, Clark et al. 1985, Scott et al. 1984, Watts et al. 1987, Scott and Weaver 1989). In addition, Rosen (1987) recently summarized information on vertical transmission of alphaviruses by mosquitoes and concluded that there is no convincing evidence that mosquitoes transmit any alphaviruses vertically.

Our vertical transmission studies are not definitive, however, because we only examined progeny from the first egg batch. Miller et al. (1979) reported that orally infected *Ae. triseriatus* (Say) did not transmit La Crosse virus to their progeny until the second gonotrophic cycle. In vertical transmission studies with flaviviruses, Rosen and coworkers (Rosen 1988, Rosen et al. 1989) have discarded the first egg batch, concentrating their efforts on subsequent ovipositionings in order to test their hypothesis that vertical transmission occurs when the fully formed egg is oviposited.

The so-called "eclipse phase" we report here for titration of *Ae. albopictus* (Fig. 1) was described earlier by Chamberlain et al. (1954b) for whole *Ae. aegypti* (Linn.) and *Ae. triseriatus*

infected with EEE virus. Studies on the replication of EEE virus in *Cs. melanura* showed no drop in titer during the first 2 days of infection (Scott et al. 1984). Presumably, EEE virus replicates and disseminates so rapidly in *Cs. melanura* that at the time intervals studied, the eclipse phase was not detectable (Scott et al. 1984, Scott and Burrage 1984).

Our fluorescent antibody results (Table 1) similarly support the conclusion that EEE virus does not disseminate as rapidly in *Ae. albopictus* as in *Cs. melanura*, which were examined in previous studies and that had imbibed a similar or lower EEE virus dose (Scott et al. 1984, Scott and Burrage 1984, Weaver et al. 1988, Scott and Weaver 1989). Replication of EEE virus in *Ae. albopictus* seems to lag about 1–2 days behind the same process in *Cs. melanura*.

After a mosquito imbibes viremic blood, the initial site of virus replication is assumed to be in epithelial cells of the posterior midgut (Chamberlain and Sudia 1961, Hardy et al. 1983, Scott and Weaver 1989). Virus then disseminates to the hemocoel, and with EEE virus in *Cs. melanura*, replication appears to take place in the abdominal and thoracic fat body before salivary glands are infected (Scott and Weaver 1989). Because the organ in *Ae. albopictus* containing the second highest percentage of detectable antigen was the abdominal fat body (Table 1), a process similar to that described in *Cs. melanura* of secondary replication in fat body may also occur in this *Aedes* species.

Results from our studies on virus replication in mosquitoes, transmission efficiency and detection of antigen in mosquito organs support the concept of a dissemination barrier (Chamberlain and Sudia 1961, Kramer et al. 1981) to EEE virus in *Ae. albopictus*. After 8–15 days of EI, all the *Ae. albopictus* we examined by virus titration (Fig. 1) and fluorescent antibody techniques (Table 1) were infected with EEE virus. However, in limited transmission studies fewer than 60% of the mosquitoes transmitted virus. In our fluorescent antibody study, virus did not appear to have disseminated from all midguts examined until after 15 days of EI. Results from fluorescent antibody studies suggest that dissemination of virus from the gut is slow but will eventually occur and that the principal barrier to dissemination and transmission of virus is infection of the salivary glands. It would be worthwhile, however, to carry out similar studies for a longer period of time to determine if the percentage of *Ae. albopictus* transmitting EEE virus increased as the duration of the EI period was increased (Chamberlain and Sudia 1961).

We have shown that if the Houston strain of *Ae. albopictus* was to imbibe viremic blood—the titers we exposed mosquitoes to are representative of a natural avian infection (Kissling et al. 1954, Scott and Olson 1986, Scott and Weaver 1989)—that mosquito infection and transmission could occur. However, other factors such as *Ae. albopictus* blood-feeding behavior, survival and fluctuations in population densities need to be assessed in field studies and incorporated into the analysis before the vectorial capacity (MacDonald 1957, Garrett-Jones and Shidraw 1969), and thus, the epidemiological importance of this mosquito in EEE virus epidemic transmission can be determined.

ACKNOWLEDGMENTS

This research was supported by The National Institutes of Health (grants A122119 and Al26787) and the Maryland Agricultural Experiment Station. Judith Grumstrup-Scott, Ronald A. Ward and two anonymous reviewers improved this manuscript with their constructive comments. In conducting the research described in this report, the investigators adhered to the *Guide for the care and use of laboratory animals*, as published by the Institute of Laboratory Animal Research, National Research Council.

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