Togavirus-Associated Pathologic Changes in the Midgut of a Natural Mosquito Vector†

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Received 3 November 1987/Accepted 8 March 1988

Arthropod-borne viruses were not previously believed to cause discernible pathologic changes in their natural mosquito vectors. We report cytopathologic lesions in the midgut of the mosquito, Culex melanura, 2 to 5 days after oral infection with eastern equine encephalomyelitis virus. Sloughing of densely stained, heavily infected epithelial cells into the midgut lumen was observed by light and transmission electron microscopy, along with degeneration of cells within the epithelium. Pathological changes in midgut epithelial cells sometimes included loss of brush border and basal lamina integrity. Disruption of the midgut basal lamina could result in bypassing of barriers to virus dissemination within the mosquito and allow rapid transmission to occur. Alternatively, luminal sloughing of heavily infected midgut epithelial cells may serve to modulate mosquito infections. These findings challenge previous beliefs regarding the benign nature of arbovirus-invertebrate host relationships.

Traditional views hold that arthropod-borne viruses (arboviruses) cause severe pathologic changes in many vertebrate hosts but no discernible pathologic changes in vector mosquitoes (4, 7, 11, 24). Several studies using transmission electron microscopy (TEM) have yielded no evidence of cytopathologic changes in mosquitoes infected with alphaviruses (15, 29, 30) and flaviviruses (31). However, two reports of pathologic changes in vectors associated with alphavirus infection are found in the literature. (i) Cytopathologic changes in salivary glands of Aedes aegypti infected with Semliki Forest virus was reported (18). The significance of this finding is questionable, however, because A. aegypti is not known to transmit that virus in nature (3). (ii) Houk et al. (13) reported vacuolization in midgut epithelial cells of A. dorsalis infected with western equine encephalomyelitis virus, as well as luminal extension of an infected cell. However, because Houk et al. (13) did not examine uninfected control mosquitoes, interpretation of these results is problematic and their significance is unknown.

Although arbovirus-induced cytopathology in a natural vector mosquito has not been documented, a growing body of evidence suggests that virus-associated deleterious effects are manifested as reduced blood feeding success (10, 27), decreased adult and larval survival (7a), delayed larval development (2, 25, 28), and reduced fecundity (2, 7a, 25). The virus-induced alterations responsible for these effects remain undescibed.

Eastern equine encephalomyelitis (EEE) virus (Togaviridae: Alphavirus) is transmitted among passerine birds in North American enzootic foci by the mosquito vector Culex melanura (9). Virus replication is initiated in mosquitoes when the posterior midgut becomes infected after blood feeding on a viremic bird (see Fig. 1). Within 2 to 3 days, virus disseminates via the hemocoeel to a variety of target organs, including the salivary glands (21, 22). Transmission to a vertebrate host, via saliva during feeding, may occur within 3 days of an infectious blood meal (21). This rapid dissemination and transmission exceeds rates reported for most other arboviruses (7, 11) and may be important in natural EEE virus maintenance.

During ultrastructural studies designed to elucidate the rapid dissemination and transmission process of EEE virus in its invertebrate host, we detected cytopathologic changes in midgut epithelia of infected mosquitoes. We report here the first well-documented evidence that arboviruses cause cytopathologic changes in their mosquito vectors.

MATERIALS AND METHODS

Virus infections. EEE virus strain ME77132, a natural 1977 C. melanura mosquito isolate from Massachusetts, was used for bird infections. This strain was passed once in Culex quinquefasciatus mosquitoes and twice in C6/36 mosquito cells before inoculation of birds. Some C. melanura mosquitoes used for experimental infections were derived from a colony established in 1967 from adult females collected in Farmington, Conn. (22); others were field collected during 1986 and 1987 at an enzootic focus of EEE virus in the Pocomoke Swamp in Maryland (20). Mosquitoes were reared and maintained as previously described (22). Baby chicks (Gallus gallus) or adult house sparrows (Passer domesticus) were infected by intramuscular inoculation with ca. 10⁶ baby hamster kidney (BHK) cell 50% tissue culture infective doses (TCID₅₀) of virus. After 24 h of incubation, birds were restrained and exposed to 7- to 14-day-old C. melanura mosquitoes in 3.8-liter plastic cages. At 1 h later, all mosquitoes were immobilized by chilling, and fully engorged individuals were separated from partially or unengorged specimens; the latter were discarded. The remaining mosquitoes were incubated in cages at 24 to 26°C and 80% relative humidity and given 5% aqueous sucrose. Control mosquitoes, allowed to engorge on uninfected birds, were identically prepared. Birds were bled immediately before and after mosquito exposure to estimate blood meal

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virus titers as the geometric mean of pre- and postexposure values.

**Fixation and microscopy.** At selected intervals after engorgement, control and experimental mosquitoes were fixed for microscopic examination (see Table 1). Individual mosquitoes were aspirated from cages, immobilized by chilling, and intrathoracically inoculated with ca. 0.5 μl of the following fixative at 5°C with glass pipettes pulled to fine tips (19): 4.0% paraformaldehyde-0.8% glutaraldehyde-0.1 M sodium cacodylate (pH 7.3)-0.001 M calcium chloride. The legs and wings were then removed, and the last two abdominal segments were severed with a razor blade. The head and abdomen were next severed from the thorax, and these three segments were immersed in fixative at 5°C for 10 h or more. The abdomen was later cut in half cross-sectionally and returned to the fixative for 4 h or more. Mosquito segments were postfixed in 1% aqueous osmium tetroxide for 8 to 12 h at 5°C and in 0.5% methanolic (30%) aqueous uranyl acetate for 8 to 12 h at 5°C. After dehydration in a graded series of acetone, segments were embedded in Spurr low-viscosity resin. After polymerization, 0.5- to 1.0-μm sections were cut with glass knives and stained with toluidine blue for light microscopy. At least four cross-sectional planes were sampled for each midgut, and one or more planes through at least three salivary gland lobes were evaluated. Ultrathin sections of 60 to 90 nm were cut with a diamond knife and stained with lead citrate for TEM. Sections were viewed and photographed on a Zeiss EM10 TEM at 60- to 80-kV electron acceleration.

**Virus assay.** Virus titers of bird blood were determined by assay of serial 10-fold dilutions in BHK cell cultures as previously described (22).

### RESULTS

Geometric means of pre- and postfeed virus titers of infected birds ranged from 10^5.2 to 10^7.7 TCID₅₀/ml. These mosquito oral doses are grouped into three categories, low, moderate, and high, for ease of discussion (Table 1).

Alterations in the appearance of anterior and posterior midgut epithelial cells were noted in all mosquitoes examined 2 to 5 days after they imbibed an infectious blood meal of high or moderate virus titer from chicks and sparrows. Control mosquitoes, those that ingested small virus doses, and all infected mosquitoes fixed before 2 or after 5 days of incubation showed normal midgut histology and ultrastructure. The occurrence of virus in the salivary glands of all mosquitoes examined after 7 to 14 days of incubation suggested that infection rates were ca. 100% for all experiments.

In mosquitoes fixed 2 to 5 days after they ingested blood meals containing moderate or high titers of EEE virus, light microscopy revealed dark-staining cells in the midgut lumina of all abdomens examined. Of all uninfected control mosquitoes examined for the corresponding intervals, only one contained a single luminal cell (day 4). Examination of ultrathin sections by TEM revealed electron-dense cells in various stages of degeneration, extending from the epithelium into the lumen (Fig. 1 and 2). Large numbers of intracellular virus particles, sometimes in paracrystalline arrays, were observed within most sections of these luminal cells (Fig. 2, inset). Serial sections of luminal cells that displayed no virus in the first plane examined revealed that all contained virus in other sections.

Ultrastructurally, most luminal cells showed features of differentiated midgut epithelial absorptive cells, including a brush border. None contained secretory granules characteristic of midgut endocrine cells (5). Serial 1-μm sections revealed that most of these cells were completely detached from the midgut epithelium. Detached cells invariably lay between the intact epithelium and the peritrophic membrane.

Degenerating cells were also detected within infected anterior and posterior midgut epithelia on days 2 to 5 by light and electron microscopy. These cells were usually light

**FIG. 1.** Representation of mosquito internal anatomy, including the location of the midgut and salivary glands. Abbreviations: M. midgut; SG, salivary glands; H, hemocoel; MV, microvilli; NU, nucleus; BL, basal lamina; BR, basal labyrinth.

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<th>Virus titer in blood</th>
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* High, 10^7.7 to 10^10.7 BHK TCID₅₀; Medium, 10^5.7 to 10^8.0 TCID₅₀; Low, 10^5.2 TCID₅₀; control, no virus.

a Not done.
staining and electron translucent (Fig. 3). Nuclei appeared vesicular, and cytoplasmic density was markedly reduced relative to that of adjacent epithelial cells. Virus particles were detected in all of the degenerating cells examined. In some cases, cellular degeneration was accompanied by disruption of the midgut basal lamina (Fig. 3). In these instances, virus within cytoplasmic debris of degenerating epithelial cells was in direct contact with the mosquito hemocoel (Fig. 3, inset). Cytopathologic changes were not detected in regenerative cells when these could be identified on the basis morphology.

Other observed cytological abnormalities of the midgut included disruption of posterior midgut epithelial cytoplasm associated with large numbers of inter- and intracellular virus particles (Fig. 4). Particle-filled intercellular and cytoplasmic spaces of up to 5 μm occasionally extended through the brush border and continuous junction, leaving only the basal lamina separating the hemocoel from the midgut lumen. Control mosquitoes also contained intercellular spaces, but these were always less than 1 μm wide in thin sections and were restricted to the basal three-fourths of the epithelium. Electron-dense, amorphous particles occasionally extended from the lumen into intra- and intercellular spaces of heavily infected midgut epithelial cells (Fig. 4). These particles were also seen in large numbers in the periphery of the digesting blood meals of control and infected mosquitoes 2 to 4 days after engorgement. However, none were detected within the epithelia of control mosquitoes. X-ray microanalysis of unstained, adjacent (to Fig. 4) thin sections revealed that these particles contained large amounts of iron.

Cytopathologic changes were not detected in salivary glands of infected mosquitoes. However, examination of salivary gland acinar cells and adjacent thoracic fat body trophocytes revealed a novel form of virus morphogenesis in one mosquito fixed on day 3 of infection. Large numbers of nucleocapsids, measuring 27 to 33 nm in diameter, surrounded pleomorphic whorls of cytoplasmic membranous inclusions in several contiguous cells (Fig. 5). These inclusions were not seen in uninfected cells of experimentally infected or control mosquitoes. Cells containing these inclusions usually displayed enveloped virus particles maturing by budding through the plasma membrane, as seen in most of the mosquito tissues examined. Some midgut endocrine cells also contained smaller accumulations of nucleocapsids lining membranous inclusions, as did one neuroendocrine cell adjacent to the posterior midgut.

DISCUSSION

Our findings of degenerating cells in the lumina and epithelia of mosquito midguts and lack of similar lesions in uninfected control mosquitoes demonstrate that cytopathologic changes accompany EEE virus infection of its enzootic
vector, C. melanura. This is the first conclusive evidence of arbovirus-associated pathologic changes in a natural mosquito vector. The timing of these cytopathologic changes parallels maximum titers of infectious EEE virus in mosquito midguts as previously reported (22) and is dose dependent. Although our findings of midgut cytopathologic changes were limited to laboratory infections with medium to high oral doses from chicks and house sparrows, natural vertebrate amplifying hosts develop comparable viremia levels. For example, the viremic response of a sentinel bobwhite quail (Colinus virginianus) naturally infected in the Pocomoke Swamp during August 1985 (T. W. Scott, unpublished data) was similar to the responses of experimentally infected chicks and sparrows (23). This information, coupled with the present demonstration of cytopathologic changes in experimentally infected mosquitoes collected from the wild, suggests that EEE virus-induced pathologic changes affect C. melanura populations in natural, enzootic foci. The potential significance of this phenomenon in the epidemiology of EEE virus warrants further study.

Two types of cytopathologic changes were seen in midguts of infected mosquitoes. (i) Condensation of infected epithelial cells was observed within and projecting into the midgut lumen, followed by degeneration (Fig. 2). (ii) Swelling and necrosis of infected cells occurred within the epithelium (Fig. 3). Increased cytoplasmic density and accumulation of virus particles has been described as characteristic of alphavirus-induced cytopathologic changes in vertebrate cell culture (1), whereas cellular swelling accompanies necrosis in a variety of animal tissues (6). Whether these represent two distinct types of cytopathologic change or different forms of expression of a single process remains unclear. Houk et al. (13) have suggested that a gradation in cell alteration may exist when A. dorsalis is infected with western equine encephalomyelitis virus.

In the present study, dense luminal midgut cells invariably revealed virus particles when serial sections were examined, suggesting that condensation and sloughing occurs in response to virus replication. The lack of sloughed cells in uninfected control midguts indicates that this alteration was associated with EEE virus replication; our data are inconsistent with the interpretation that EEE virus replicates excessively in epithelial cells undergoing sloughing as part of normal cell turnover. The location of all detached cells between the intact epithelium and peritrophic membrane suggests that sloughing occurred 8 h or more after the bloodmeal, when the peritrophic membrane begins forming in C. melanura (S. C. Weaver, unpublished data).

Our study did not address the question of whether infected cells become detached after cytopathic effects or in response to initial viral infection. The location of most electron-dense cells extending into the lumen suggests that condensation of

FIG. 3. TEM of infected, degenerating epithelial cells in posterior midgut of C. melanura 60 h after moderate-titer infection with EEE virus. Note the vesicular-appearing nucleus (N) and reduced cytoplasmic density of degenerating cells compared with normal epithelial cells (E). The basal lamina is disrupted and terminates adjacent to degenerating cells (B). Magnification, ×5,000. The inset shows a high-magnification view of virions within cytoplasmic debris in an area of degeneration (arrow) exposed directly to the hemocoel, above. Magnification, ×55,000.
damaged cells is a response to increased contact with the digestive luminal environment; infected, damaged cells with less luminal contact may simply swell and degenerate (Fig. 3).

Disruption of epithelial brush border and cytoplasm was exclusively associated with EEE virus infection and was often accompanied by large numbers of virus particles (Fig. 4). The dense, iron-containing particles seen within inter- and intracellular disruptions are apparently a product of hemoglobin digestion, since these occur in all blood meals 2 to 4 days after engorgement. The lack of these particles within the epithelia of control mosquitoes suggests that they entered the epithelia from the lumina after virus-induced alterations. Houk and Hardy (12) have reported leakage of a blood meal component into intercellular spaces of Culex tarsalis midguts after engorgement on uninfected blood. However, continuous junctions remained intact or only slightly widened, in contrast to the dramatic disruptions we report associated with EEE virus infection of C. melanura. Possible physiological effects of this midgut disruption deserve further study.

The large numbers of EEE virus nucleocapsids we observed associated with membranous inclusions in salivary gland acinar cells and fat body trophocytes were restricted to a single infected mosquito. These structures were found adjacent to large arrays of rough endoplasmic reticulum and in some respects resembled cisternae of endoplasmic reticulum with collapsed lumina. Identical structures are not described in the literature. They bear superficial resemblance to the cytoplastic “virus factories” hypothesized by Brown and Condrey (4) to protect mosquito cells from cytopathic effects of alphavirus maturation by plasma membrane budding. However, the inclusions we describe contain double membranes with no luminal spaces for budding of nascent virus. In the present study, maturation of enveloped virus was observed only at the plasma membrane. Cytoplasmic virus factories have never been identified in the ultrastructural studies we have conducted on mosquitoes infected with EEE, western equine encephalomyelitis, or Venezuelan equine encephalomyelitis virus (S. C. Weaver, personal observations). We therefore have no evidence from in vivo mosquito infections to support the virus factory hypothesis of Brown and Condrey.

In our study, many obviously infected midgut epithelial cells showed no apparent cytopathologic changes, whereas others were undergoing necrosis. Two possible explanations for this variation are differences among midgut epithelial cells or within experimental populations of EEE viruses.

Some investigators have reported that C6/36 (a cell line established from A. albopictus mosquitoes) cells undergo cytopathologic changes after togavirus infection, whereas others have reported no discernible alterations (4). Tooker

FIG. 4. TEM of posterior midgut of high-titer-infected C. melanura after 72 h of incubation. Large numbers of virions are seen in basal intercellular spaces (v). Electron-dense, amorphous particles (p) extend through the brush border, below, into apical and basal cytoplasm. These articles contain large amounts of iron and are believed to be a digestive product of blood meal hemoglobin. Magnification, ×5,400. The inset shows a high-magnification view of extracellular, round virions (above) and amorphous iron-containing particles (below). Magnification, ×60,000.
FIG. 5. Accumulations of EEE virus nucleocapsids lining cytoplasmic membranous inclusions in a salivary gland acinar cell of C. melanura 3 days after infection. Magnification, ×60,000.
and Kennedy (26) demonstrated that clones of C6/36 cells responded differently to infection with Semliki Forest virus; most clones gave low yields of virus with no discernible cytopathologic changes, whereas 30% gave high yields with moderate-to-severe cytopathologic changes. Selection of low-virus-producing cell lines followed infection of mixed cell populations, resulting in modulation and persistent infections.

Modulation of EEE virus replication is known to occur in C. melanura; previous studies have shown that EEE virus infectious titers decline about 1 week after per os infection (22). Therefore, the findings of Tooker and Kennedy (26) could be relevant to in vivo mosquito infections. Differential responses to EEE virus infection among midgut epithelial cells, resulting in necrosis and sloughing of high-virus-producing cells, could alter midgut cell populations and thus serve to modulate virus replication in a mosquito vector. In fact, Kramer et al. (14) hypothesized that midgut epithelial cell sloughing might occur in Culex tarsalis infected with western equine encephalomyelitis virus and might be involved in modulation of virus replication in that species.

An alternative explanation for variability in midgut cellular responses to infection could relate to variation within our experimental EEE virus populations. Because arboviruses are known to exist in (low-passage isolates) as mixtures of genetically discrete viruses (8, 17), selection from preexisting heterogeneity could occur during mosquito infection. Possible outcomes of such selection could include (i) selection for virus attenuation if pathology caused by virulent genotypes interferes with the ability of infected mosquitoes to transmit these viruses or (ii) selection for virulent viruses which cause midgut epithelial cell necrosis and basal lamina disruption and thereby outcompete less virulent forms by disseminating more rapidly to salivary glands. Evolutionary trends that favor virulence, accompanied by enhanced transmission, have been reported for other vertebrate pathogens (16).

In summary, the demonstration of EEE virus-associated pathologic changes in a vector mosquito is important for several reasons. (i) It challenges previous beliefs (4, 7, 11, 24) regarding the benign nature of these parasite-host interactions and suggests that these viruses affect mosquitoes in nature. This provides additional evidence that commensalism is not necessarily the end result of well-developed parasite-host interactions (16). (ii) Our results confirm previous findings that alphaviruses can cause cytopathologic changes in certain mosquito cells in vitro and are consistent with certain hypotheses (26) concerning modulation mechanisms for alphavirus replication in mosquito cell cultures. (iii) Our findings suggest that midgut pathologic changes are important in promoting dissemination of EEE virus to certain target organs during mosquito infections and thus could affect virus transmission, epidemiology, and evolution.

ACKNOWLEDGMENTS

This research received support from Public Health Service grants AI20675 and AI22139 from the National Institutes of Health, the Avrum R. Gudelsky Research Fund, and the Maryland Agricultural Experiment Station.

We thank Judith Grumstrup-Scott and E. W. Cupp for editorial assistance, and John A. Terzakis and Timothy K. Maugel for help with ultrastructural interpretation. Aileen Hsu drew Fig. 1.

LITERATURE CITED