Infectivity and Pathogenicity of Nodamura Virus for Mosquitoes

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SUMMARY

Nodamura virus multiplied and caused paralysis and death when injected into the thoraces of adult Aedes albopictus and Toxorhynchites amboinensis mosquitoes but not when similarly injected into Culex quinquefasciatus adults. A. albopictus also became infected after ingesting a Nodamura virus suspension or after immersion in a virus suspension as larvae, but they did not die. Head squash preparations of the infected insects, examined by indirect fluorescent antibody technique, showed large amounts of Nodamura virus antigen in the brain regardless of the mode of infection. Nodamura virus was isolated from and titrated in mosquito cell (AP-61) cultures. Comparative titrations indicate that this method of assay is more sensitive than intracerebral inoculation of infant mice.

INTRODUCTION

Nodamura virus was isolated in 1956 from a single pool of Culex tritaeniorhynchus mosquitoes collected in Japan (Scherer & Hurlbut, 1967). This small RNA virus is unusual because it is lethal both for a vertebrate and for insects. When inoculated into infant mice, Nodamura virus produces flaccid ascending paralysis and death (Scherer et al. 1968). It also kills adult honey bees (Apis mellifera) and wax moth larvae (Galleria mellonella), when injected into their haemolymph (Bailey & Scott, 1973).

Because Nodamura virus was originally recovered from C. tritaeniorhynchus, can be transmitted to mice by Aedes aegypti (Scherer & Hurlbut, 1967) and will replicate in A. aegypti and A. albopictus cell cultures (Bailey et al. 1975), it has generally been assumed that the virus is mosquito-borne. In order to learn more about the behaviour of this virus in its presumed insect host, a series of experiments was done using three species of mosquitoes and several different routes of infection. This paper reports the results of these studies and describes a method for assaying Nodamura virus in mosquito cell cultures.

METHODS

Virus. Nodamura virus (strain Mag 115) at the thirteenth mouse passage level was obtained from Dr William F. Scherer. After the virus was received, it was passed once more in infant mice by intracerebral inoculation. Brains and limbs from the dead or moribund animals were triturated in phosphate-buffered saline containing 0.5% gelatin (PBS-G), to prepare a 10% suspension which was stored frozen at −70°C and used as virus stock to infect mosquitoes. The titre of this stock was 10^7.4 infant mouse LD_{50} units per ml as determined by intracerebral inoculation.

Mosquitoes. The three species of mosquitoes (Aedes albopictus, Toxorhynchites amboinensis and Culex quinquefasciatus) used in these studies were from colonies maintained at the...
Pacific Research Unit. The colonies were established in 1971, 1975 and 1978, respectively, from specimens collected on the island of Oahu, Hawaii. Mosquitoes were reared by standard laboratory techniques (Gerberg, 1970) in an insectary maintained at approx. 28 °C with 16 h of light and 8 h of darkness.

**Infection of mosquitoes.** Mosquitoes were infected with Nodamura virus either by intrathoracic injection or by feeding, or by immersion of larvae in a concentrated virus suspension. Intrathoracic inoculation was done using a method described by Rosen & Gubler (1974). Feeding was done by allowing adult female mosquitoes, previously fasted for 24 h, to engorge on a blood–virus–sugar suspension consisting of equal volumes of washed human erythrocytes, heated calf serum with 10% sucrose and the Nodamura virus pool. Only females that fully engorged were kept. Infection by immersion was done by placing second and third stage larvae of *A. albopictus* in a concentrated virus suspension for 24 h.

**Preparation of immune ascitic fluid.** Specific immune ascitic fluid was prepared in adult mice, using the Nodamura virus stock as antigen. The first injection of virus antigen (0.1 ml) was given subcutaneously. The second and third injections, given intraperitoneally 21 and 35 days later, respectively, consisted of 0.5 ml of a 1:1 mixture of virus and Freund’s complete adjuvant. The animals also received 0.1 ml of a suspension of Sarcoma-180 cells intraperitoneally with the final immunization. Ascitic fluids were collected as they accumulated.

**Cell cultures.** The *Aedes pseudoscutellaris* (AP-61) cell line was grown as described by Varma *et al.* (1974) in standard (16 × 125 mm) screw-cap tissue culture tubes at 28 °C.

**Virus assay.** Nodamura virus was detected in the brains of individual adult mosquitoes by an indirect fluorescent antibody technique (IFAT) performed on head squashes of the insects and described by Kuberski & Rosen (1977). The virus was assayed in tube cultures of AP-61 cells. The specimens to be tested were triturated in sterile tissue grinders containing 2.0 ml of PBS-G with 30% heated (56 °C for 30 min) foetal bovine serum. After centrifugation (3000 g for 30 min), 0.1 ml of the supernatant fluid was inoculated into duplicate tubes of AP-61 cells. For titrations, 0.1 ml of each of a series of tenfold dilutions of the extract in PBS-G was inoculated into each of four tube cultures. After adsorption for 2 h, maintenance medium was added to the cultures and they were incubated at 28 °C for 8 to 10 days. Suspensions of the inoculated AP-61 cells were then prepared, stained and examined by IFAT for the presence of Nodamura virus antigen (Tesh, 1979). Head squashes of uninoculated laboratory-reared mosquitoes and non-infected AP-61 cells were included in all tests as controls. Virus titres were calculated by the method of Reed & Muench (1938).

**RESULTS**

**Infection of mosquito cell cultures**

In preliminary studies, AP-61 cell cultures were inoculated with various dilutions of the Nodamura virus stock. The cells were examined by IFAT for the presence of virus antigen after 7 to 10 days. Although no c.e.p.e. was observed, approx. 25 to 30% of the cells contained Nodamura virus antigen. At high virus dilutions (≥ 10^-5.5), a smaller percentage of cells was antigen positive. The infected cells occurred singly or in clusters, with the virus antigen confined mainly to the cytoplasm (Fig. 1). Fluorescence was not observed in controls. Simultaneous titrations of the Nodamura virus stock in tube cultures of AP-61 cells and by intracerebral inoculation of newborn mice indicated 10^8.5 50% tissue culture infectious dose (TCID_{50}) and 10^7.4 LD_{50} per ml, respectively. Accordingly, the AP-61 cells were used for the assay of virus in mosquitoes.
Nodamura virus in mosquitoes

Fig. 1. AP-6t cells infected with Nodamura virus and examined by IFAT. The cluster in the foreground contains both antigen-positive and antigen-negative cells. Magnification approx. ×320.

Fig. 2. Head squash preparation of a paralysed mosquito (A. albopictus) which was inoculated with Nodamura virus and examined by IFAT. Nodamura virus antigen is spread diffusely throughout the brain. Magnification approx. ×320.

Infection of mosquitoes by intrathoracic injection

In replicate experiments, adult female A. albopictus, T. amboinensis and C. quinquefasciatus were each inoculated with 0.17 μl of the undiluted Nodamura virus stock. Ten days later, the C. quinquefasciatus were alive and well, but all of the other two groups died between the sixth and tenth day after inoculation. Shortly before death they manifested loss
of balance, inability to fly and eventually paralysis. Examination of head squashes of dead or paralysed mosquitoes revealed large amounts of virus antigen in the brain (Fig. 2). The antigen appeared to be both intracellular and extracellular, in contrast to its distribution in the infected AP-61 cell cultures (Fig. 1).

Serial tenfold dilutions of the Nodamura virus stock were then inoculated into adult female *A. albopictus* using ten insects per dilution. The mosquito LD₉₀ by this technique was 10⁻⁴·₇ per ml, very similar to the titre in AP-61 cells.

The ability of Nodamura virus to replicate in *A. albopictus*, *Toxorhynchites amboinensis* and *Culex quinquefasciatus* was compared. Ten adult female mosquitoes of each species were inoculated with 10⁻³·₇ TCID₉₀ of Nodamura virus. On the sixth day after inoculation, all of the mosquitoes were killed by freezing and five females of each species were titrated individually in tube cultures of AP-61 cells. The results are shown in Table I.

To determine whether the relatively small amounts of virus present in *C. quinquefasciatus* after 6 days were due to low levels of virus replication or to persistence of the original inoculum, an experiment was done to determine the stability of Nodamura virus at ambient temperature. A vial of the original Nodamura virus pool was divided into two parts. The first portion was titrated immediately after thawing; the second was held for 31 days at 28 °C before testing. The virus titres of these two specimens were 10⁻⁵·₅ and 10⁻⁵·₅ TCID₉₀ per ml, respectively, as assayed in AP-61 cells.

### Oral infection of mosquitoes

Sixteen out of 76 adult female *A. albopictus* that were fed on a blood–virus–sugar suspension containing approx. 10⁻⁵·₀ TCID₉₀ of Nodamura virus per ml gave positive results by IFAT 11 days after feeding. Except for a slight reduction in the intensity of fluorescence, the distribution and appearance of virus antigen in this group of mosquitoes was similar to that observed in the parenterally infected group (Fig. 3). However, no mortality was noted in the orally infected group.

### Infection of larvae by immersion in a virus suspension

Approximately 100 second and third stage *A. albopictus* larvae were kept for 24 h in water containing 10⁻⁶·₀ TCID₉₀ of Nodamura virus per ml. They were then rinsed repeatedly with tap water and transferred to rearing pans. Fourteen days later, 22 of 99 (22·₂ %) head squash preparations of the resulting adults contained Nodamura virus antigen. The amount and intensity of fluorescence in the brains of these insects was similar to that observed in the parenterally infected group (Fig. 3). However, no mortality was noted in the orally infected group.

### Attempt to demonstrate transovarial virus transmission

Three experiments were done to determine whether Nodamura virus was transovarially transmitted in mosquitoes. In the first experiment, 100 *A. albopictus* females were injected with approx. 10⁻₄·₅ TCID₉₀ of virus. Five days after inoculation, the mosquitoes were allowed

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**Table I. Nodamura virus titres in mosquitoes 6 days after inoculation with 10⁻³·₇ TCID₉₀ of virus**

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Number of mosquitoes sampled</th>
<th>Range of virus titres in mosquitoes sampled</th>
<th>Mean virus titre</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Toxorhynchites amboinensis</em></td>
<td>5</td>
<td>6·₈–₈·₈*</td>
<td>7·₆*</td>
</tr>
<tr>
<td><em>Aedes albopictus</em></td>
<td>5</td>
<td>6·₃–₇·₆</td>
<td>7·₂</td>
</tr>
<tr>
<td><em>Culex quinquefasciatus</em></td>
<td>5</td>
<td>1·₈–₂·₈</td>
<td>2·₄</td>
</tr>
</tbody>
</table>

* Virus titres expressed as log TCID₉₀ per insect.
Fig. 3. Head squash preparation of an *A. albopictus* female which had ingested a Nodamura virus suspension 11 days previously, examined by IFAT. Although slightly less virus antigen was present in the brain of this insect than in the specimen shown in Fig. 2, the distribution of the antigen was similar. Magnification approx. ×320.

...to feed on a normal mouse. By the tenth day, most of the parent mosquitoes were dead, although a number of the insects laid eggs before dying. These eggs were hatched and the F₁ larvae reared to adults. A total of 740 F₁ *A. albopictus* adults were divided into eight groups which were tested in tube cultures of AP-6₁ cells. No virus was detected.

A second and similar experiment was done using *C. quinquefasciatus*. A total of 414 F₁ adults was tested individually by IFAT or extracts of groups were tested in AP-6₁ cells. Again, no virus was detected.

In the third experiment, eggs were collected from a group of 76 *A. albopictus* females which had fed on a blood–virus–sugar suspension. A total of 1000 F₁ adult *A. albopictus* from the second ovarian cycle of the orally infected parents was tested for Nodamura virus in AP-6₁ cell cultures. Although 21.1% of the parent mosquitoes were infected, no virus was detected in any of their progeny.

**DISCUSSION**

The standard method for isolation and titration of Nodamura virus has been intracerebral inoculation of infant mice. Use of AP-6₁ cells for assay of the virus eliminates the inconvenience and expense of handling large numbers of mice. Furthermore, the AP-6₁ cells provide a more sensitive assay system for Nodamura virus than infant mice.

In the original description of Nodamura virus by Scherer & Hurlbut (1967) there was some doubt whether the virus multiplied in *A. aegypti*, although the mosquitoes transmitted the agent, possibly mechanically, when they bit mice. The results described here indicate that the virus replicates well in both *A. albopictus* and *T. amboinensis* but poorly or not at all in *C. quinquefasciatus* (Table 1). The low virus titre in the latter species was possibly no
more than the residue of the original inoculum, some of which could have persisted for at least 6 days. The fact that *C. quinquefasciatus* survived parenteral injection of the virus whereas *A. albopictus* and *T. amboinensis* were killed also supports this hypothesis.

The lethal effects of Nodamura virus infection on mosquitoes has not been reported previously, although Bailey & Scott (1973) found that the virus produced paralysis and death when parenterally injected in honey-bees and wax moth larvae. Although intrathoracic injection of Nodamura virus was lethal to *A. albopictus*, those mosquitoes infected by feeding or by immersion were not killed. This was surprising since the amount of virus antigen observed in the brains of infected mosquitoes in the three groups was similar (Fig. 2 and 3). Some of the insects infected by feeding or immersion might have died if they had been kept longer, but it is also possible that the pathogenesis of Nodamura virus in *A. albopictus* differs between oral and parenteral infection.

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REFERENCES


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