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The Multiplication of Nodamura Virus in Insect and Mammalian Cell Cultures

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SUMMARY

Nodamura virus multiplied in mosquito cell lines, as determined by infectivity assays in adult honey bees (*Apis mellifera*) and wax moth larvae (*Galleria mellonella*). Titres of more than 10^7 and 10^5 bee LD_{50} /ml were obtained in culture fluids of *Aedes albopictus* and *Aedes aegypti* cells respectively after 10 days. Comparable titres were obtained after several months, during which the cultures were subdivided up to six times. Nodamura virus also multiplied in BHK cells and yielded titres of $10^{4.8}$ to $10^{6.6}$ mouse LD_{50}/ml and $10^{5.1}$ to $10^{7.1}$ wax moth LD_{50}/ml in culture fluid 1 to 4 days after infection. No c.p.e. was observed in infected cells.

INTRODUCTION

Nodamura virus occurs in nature as an inapparent infection of *Culex tritaeniorhynchus* in Japan (Scherer & Hurlbut, 1967). As antibodies to the virus occurred in pigs in the same locality, and as the virus is transmissible to suckling mice by infected individuals of *Aedes aegypti*, it was classified as an 'arbovirus', but with some unusual physico-chemical properties (Scherer, Verna & Richter, 1968; Newman & Brown, 1973). It also has unusual biological properties for it can fatally infect honey bees (*Apis mellifera*) and the greater wax moth (*Galleria mellonella*) (Bailey & Scott, 1973) as well as mice and so is the only virus known to be lethal both for a vertebrate and insects. This contrasts with (1) the previously described arthropod-borne viruses, which, although not necessarily harmless to their vectors, are known to cause severe disease only in vertebrates or plants, and (2) the viruses that are known to cause severe disease only in insects, and which typically have very narrow host ranges.

Nodamura virus caused no c.p.e. when inoculated into several vertebrate tissue cultures (Scherer *et al.* 1968) and we have recently found that it similarly causes no c.p.e. when inoculated into mosquito cell or BHK cell cultures. Nevertheless it multiplies in these cells and can readily be detected in them by infectivity tests with bees, wax moths or suckling mice.

METHODS

Mosquito cell cultures. Monolayers of cells of *Aedes albopictus*, from an isolate made by Singh (1967) and *Aedes aegypti*, Mill Hill Strain, isolated by the method of Singh (1967), were maintained at 30 °C in small flat-sided, glass bottles containing 5 ml of a medium of the following constitution: 87 % Leibowitz L15 medium, 3 % foetal calf serum, 10 % tryptose-phosphate broth, 200 mM-glutamine and 2 international units (iu) per ml of penicillin and of streptomycin (Porterfield & De Madrid, 1971). Half the medium was replaced at intervals of 7 to 14 days, except when otherwise indicated. Cells were subcultivated by discarding the medium and adding 0·3 ml of 2·5 % trypsin + 2·7 ml of 0·02 % EDTA in phosphate buffered saline (PBS), pH 7·3. This was left for 1 min, then discarded and the cells incubated for 2 to 5 min at 30 °C till they began to detach from the glass. Ten ml of culture fluid were then added, the bottle shaken vigorously and the suspension divided into fresh bottles and incubated at 30 °C. Monolayers of about 10^6 cells formed after about two days, when they were used for infectivity tests.

The inoculum for mosquito cell cultures was prepared by grinding heads of bees that had been killed by Nodamura virus in a 3:1 mixture of 0·01 M-phosphate buffer (pH 6·5) and diethyl ether, followed by 1 vol. of carbon tetrachloride. The aqueous layer of the extract, which contained the equivalent of 1 head/ml, was cleared at 8000*g* for 10 min, filtered through a Millipore filter (pore size 450 nm), diluted 1/100 in PBS, and 0·2 ml added to the culture medium. After 2 h at 30 °C the medium was discarded, and the cell layers washed five times, each time with 5 ml of PBS. Five ml of culture medium were then added and the culture incubated at 30 °C. Samples, each of 0·2 ml of the culture fluid, were removed at the appropriate intervals and dilutions made in phosphate buffer and injected into adult bees. Fresh culture fluid was not added to tissue cultures during the test period.

Inoculated and uninoculated cells were also cultivated on cover slips, fixed and stained with Giemsa/May–Grunwald stain.

BHK cell cultures. Monolayers of BHK 21 cells in 4 oz bottles containing 10^7 cells were washed once with PBS and then inoculated with 10 ml of purified virus (Newman & Brown, 1973) containing 10^8 mouse LD_{50}/ml. After 2 h at 37 °C the medium was discarded and the cell layers washed 3 times, each time with 10 ml of PBS. Ten ml of Eagle's basal medium were then added and the culture incubated at either 32 or 37 °C. Samples, each of 0·1 ml of the culture medium, were removed at intervals, diluted in phosphate buffer and titrated for infectivity in either suckling mice or wax moth larvae. Comparative tests of infectivity (Table 1 and 2) in wax moths and mice of the same virus preparations could not be done simultaneously so assays were begun in mice and the preparations then mixed in an equal vol. of glycerol stored at −20 °C and tested a few days later in wax moths.

Tests in insects. Adult bees were kept in small cages (Bailey, 1969), with 20 bees per cage. Each bee was anaesthetized with CO_2, injected through a dorsal abdominal intersegmental membrane with 1 μl of diluted culture fluid and then incubated at 30 °C. Bees killed by Nodamura virus died from the 8th to the 16th day after they had been injected. The head of each bee that died was extracted in 0·05 ml saline + 1 drop of diethyl ether and the extract tested by immunodiffusion against suitably diluted antiserum. The very few extracts that failed to give precipitin lines, which were single and very distinct with extracts of bees killed by Nodamura virus, were considered negative. Their number was subtracted from the number of bees that had died after injection with the relevant dilution. The LD_{50} was calculated according to the method of Reed & Muench (1938).

Wax moth larvae were injected when half-grown (about 12 mm long) and were then kept

Table 1. *Infectivities (log LD_{50}/ml) for adult bees, wax moths and mice of cell-free culture fluid from tissue cultures inoculated with Nodamura virus*

Cells	Incubation temperature (°C)	Tissue culture inoculum*	Test animal	Day 0	1	2	3	4	6	8	11	14	16	44
Aedes albopictus	30	5·0	Honey bee	< 4·0	4·5	4·8	—	5·4	6·2	7·5	7·5	7·8	8·2	7·8
BHK	32	8·0	Mouse	2·2	5·2	5·4	4·5	6·6						
			Wax moth larva	< 4·0	5·1	—	5·7	7·1						
BHK	37	8·0	Mouse	2·2	4·8	4·8	5·6	6·0						
			Wax moth larva	< 4·0	5·4	—	5·7	< 6·0						

* Expressed in log LD_{50} for the appropriate test animal/ml of culture fluid.

Table 2. *Titres of Nodamura virus in mosquito tissue cultures, bees, wax moths and mice*

Virus source	Particles	Bee LD_{50}	Wax moth LD_{50}
Aedes albopictus tissue culture cells, 4 months post-infection	10^5/cell*	—	—
A. albopictus tissue culture, cell-free supernatant fluid, 14 days post-infection	10^{10}/ml*	$10/^8$ml*	—
Aedes aegypti tissue culture, cell-free supernatant fluid, 14 days post-infection:	—	10^6/ml*	—
Honey bees (heads) killed by virus	—	$10^{8\cdot3}$/head	$10^{8\cdot4}$/head
Wax moth larvae killed by virus	$10^{11\cdot9}$/larva	—	$10^{8\cdot5}$/larva
Suckling mice killed by virus	—	—	$10^{1\cdot0}$, $10^{2\cdot4}$/mouse LD_{50}

* Approximate values.

at 30 °C in Petri dishes with food (baby cereal: wheat germ:non-fat dried milk:honey: glycerol; 8:8:4:3:3 parts by vol.). Mortality was measured after 3 or 4 weeks, when all the survivors had emerged as adult moths. Tests with wax moths took longer and were less consistent than with bees but were done in winter when bees are unsuitable for experiments.

Virus identification. Preparations were examined for virus particles in a Siemens Elmiskop I. The concentrations of particles in preparations were estimated by the method of Nixon & Fisher (1958) except that the specimens were treated with neutral potassium phosphotungstate instead of being shadowed with metal.

Immunodiffusion tests are described by Mansi (1958) were done with virus preparations using Nodamura virus antiserum prepared against the virus from bees or mice (Bailey & Scott, 1973), in agar containing 0·04 M-sodium borate buffer, pH 7·0+0·85 % NaCl+ 0·02 % sodium azide.

RESULTS

Mosquito cell cultures

The results are summarized in Tables 1 and 2. The PBS used for the fifth rinse of the *Aedes albopictus* cells after inoculation was not infective, so most of the original inoculum had been adsorbed or washed away in the previous rinses. However, even if all the inoculum had been adsorbed and subsequently eluted into the final volume of tissue culture fluid, it

could not have produced the greatly increasing infectivity of the culture fluid after the fourth day. Several other tests gave the same results, which show unequivocally that Nodamura virus multiplied in cultures of cells of *A. albopictus*. Culture fluid from uninoculated mosquito cell lines had no effect when injected into bees.

Cells certainly produced much virus from the 4th day to the 16th day and very probably for at least a month because other tests showed that the infectivity of Nodamura virus incubated for a month in tissue culture medium without cells fell to less than 10^{-3} of that of the original inoculum.

Infected cells were as readily subcultivated as healthy ones and continued to yield the same amount of virus as the original culture. For example, after cultures of *Aedes albopictus* cells that had been inoculated with virus from wax moths had been subcultivated 6 times during 4 months with 6 complete and 7 partial renewals of culture medium, their culture fluid contained at least 10^7 bee LD_{50} per ml, 14 days after their final subcultivation. Similarly, after infected cultures of *A. aegypti* cells had been subcultivated 4 times during 4 months with 4 complete and 4 partial renewals of culture medium, their culture fluid contained at least 10^5 bee LD_{50} per ml.

Cell cultures of *Aedes albopictus* and *Aedes aegypti* were equally susceptible to Nodamura virus from wax moth larvae or from bees; and wax moth larvae and bees were equally susceptible to the virus from cell cultures that had been infected with virus from either test insect. No c.p.e. was observed in cells from either species of mosquito, in culture or in stained cell films, whether inoculated with Nodamura virus from bees or wax moth larvae.

Infected mosquito cells contained more virus than the culture fluid. For example, tissue of two of the cultures of the *Aedes albopictus* cells that had been subcultivated 6 times, mentioned above, and containing 10^6 to 10^7 cells, were triturated in a small vol. of phosphate buffer + CCl_4 two days after they had been subcultivated, and the virus was then sedimented at 150000*g* for 35 min. The pellet, re-suspended in 0·1 ml buffer, contained about 10^{10} to 10^{11} virus particles which reacted strongly in immunodiffusion tests. However, the fluid from the parent cultures, similarly treated, yielded only 10^9 to 10^{10} virus particles.

BHK cell cultures

Nodamura virus multiplied in BHK cells for at least 5 days (Table 1). The last sample for titration was taken after only 4 days because by then even non-infected control cells were beginning to detach from the glass at 37 °C, although they remained attached longer at 32 °C. About the same amount of virus was produced at either temperature. No c.p.e. was observed, but by contrast with mosquito cells, BHK cells contained less virus than the culture medium during the period 1 to 4 days.

Replication of Nodamura virus in BHK cells was confirmed by the identification of radioactively labelled, newly synthesized, virus-specific RNAs 6 h after infection (J. F. E. Newman, personal observation). In these experiments BHK cells were infected with Nodamura virus and incubated at 37 °C in the presence of actinomycin D and [^{3}H]-uridine. After 6 h the RNA was extracted with SDS phenol and centrifuged on a sucrose gradient. The radioactively labelled single stranded RNAs isolated in this way had mol. wt. identical with those of the RNAs extracted from virus particles by the same method. Ribonuclease resistant replicative forms of RNA with mol. wt. approx. double those of the single stranded RNAs were also isolated from the infected cells.

The ratio of titres in mice and wax moth larvae varied considerably. There may be large differences between the sensitivities of wax moth larvae that differ only slightly in age, and the virus may have lost some infectivity at different rates in different preparations during

storage. Nevertheless, wax moth larvae were on the whole more sensitive than suckling mice, and this was confirmed in other tests when the infectivities of Nodamura virus preparations from infected mice were compared with wax moth larvae and mice (Table 2).

DISCUSSION

The multiplication of Nodamura virus without obvious c.p.e. is analogous to the multiplication of Semliki Forest virus in *Aedes aegypti* cell lines (Peleg, 1969) and of vesicular stomatitis virus in *Antheraea eucalypti* cell lines (Yang, Stoltz & Prevec, 1969). Whether as in in these instances, Nodamura virus infects only a fraction of the cells or whether it infects all of them remains to be determined. However, our results show that virus multiplied to many times the inoculated dose in *Aedes albopictus* cells, whereas the titre of Semliki Forest virus in *A. aegypti* cells decreased to less than the inoculated dose after 10 days and the yield of vesicular stomatitis virus in *A. eucalypti* was not significantly greater than in the inoculum. Moreover, as we were able to subcultivate infected mosquito cells, seemingly indefinitely, with no loss of virus titre, there is nothing to suggest that only a very small fraction of cells are susceptible and subsequently die.

The ready detection of Nodamura virus from apparently healthy BHK cell cultures raises the possibility that the virus will multiply in other mammalian cell lines. Low levels of virus multiplication in them might best be detected with bees or, more conveniently for most laboratories, wax moths, which are somewhat more sensitive than suckling mice.

The sensitivity of honey bees to Nodamura virus does not reflect a general susceptibility of this insect to injected foreign viruses. For example, we have found that viruses that kill other insects such as *Tipula* iridescent virus, with its ability to multiply in a fairly wide insect host range (Smith, 1967), denso-nucleosis virus of the greater wax moth (*Galleria mellonella*) and free virus particles of nuclear polyhedrosis viruses of the greater wax moth and of a sawfly (*Neodiprion* sp.) do not multiply when injected into bees. Similarly, viruses that kill insects do not necessarily multiply when inoculated into mosquito cell cultures. For example, acute and chronic bee-paralysis viruses do not multiply in cell cultures of *Aedes albopictus* or of *Aedes aegypti* (L. Bailey & J. S. Porterfield, unpublished observations). Therefore, the wide host range of Nodamura virus is remarkable. Nevertheless, the virus does not multiply in all insects. For example, Scherer & Hurlbut (1967) failed to detect its multiplication in bedbugs (*Cimex lectularius*) or cockroaches (*Blatta germanica*); and we have failed to show that it multiplies in crickets (*Acheta domestica*), using bees for bioassay.

The reason why Nodamura virus can infect and multiply in tissues of unexpected diversity would be relevant to explanations of host-specificity of viruses. As the sum of mol. wt. of the two Nodamura virus ribonucleic acids is only about $1{\cdot}5 \times 10^6$ (Newman & Brown, 1973) the ability to infect tissues of a wide range of species does not necessarily depend upon very much genetic information. Further studies of Nodamura virus in its very different host tissues might well give some insight into this subject.

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