Characterization of Kawino Virus, an Entero-like Virus Isolated from the Mosquito Mansonia uniformis (Diptera: Culicidae)

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SUMMARY

Some properties of a small RNA virus isolated from Mansonia uniformis are described. The virus particles have a sedimentation coefficient of about 165S, buoyant density in CsCl of 1.33 g/ml and diameter of 28 nm. They contain a single-stranded RNA which probably lacks poly (A) but is infective. Four polypeptides with mol. wt. of 33, 30, 27 and about 7 x 10^3 are present in the virus particle.

INTRODUCTION

Members of the baculovirus, iridovirus and parvovirus families, and cytoplasmic polyhedrosis viruses, have been found in various mosquito species (David, 1975). There are also two previous reports of small RNA viruses in mosquitoes, namely Nodamura virus, isolated from Culex tritaeniorhynchus mosquitoes (Scherer & Hurlbut, 1967) and a virus described as mosquito picornavirus which was isolated from larvae of Aedes taeniorhynchus infected with mosquito iridescent virus. The presence of mosquito picornavirus could only be detected in larvae infected with mosquito iridescent virus (Wagner et al., 1974). Many small RNA viruses have been isolated from other arthropods (Brown & Hull, 1973; David, 1975). However, of these viruses only those from six orders of insects have been characterized in any detail: in the Hymenoptera, five viruses of the honey bee (Newman et al., 1973b; Bailey & Woods, 1974); in the Lepidoptera, Gonometa virus and Nudaurelia virus (Longworth et al., 1973; Struthers & Hendry, 1974); in the Orthoptera, cricket paralysis virus (Reinganum et al., 1970; Reinganum, 1973); in the Coleoptera, a virus from Heteronychus arator (Longworth & Carey, 1976); and in the Diptera Nodamura virus (Murphy et al., 1970; Newman & Brown, 1973, 1976, 1978), kelp fly virus (Scotti et al., 1976) and Drosophila C virus (Jousset et al., 1977).

This report extends the information on viruses isolated from dipteran species by describing some of the properties of an entero-like virus isolated from Mansonia uniformis mosquitoes. The virus has been called Kawino, since the mosquitoes from which it was isolated were collected near the village of Kawino in the Kano Plain district of Kenya.

METHODS

Virus isolation. Kawino virus was isolated from a pool of 100 Mansonia uniformis mosquitoes, no. K2528/73, collected as part of the MRC Project based at Kisumu in Kenya. Pool K2528/73 was tested for arboviruses using techniques previously described but none was detected (Johnson et al., 1978). Using the methods previously described (Varma et al., 1975/76), a 1:5 dilution of K2528/73 in Leibovitz L-15 medium (Leibovitz, 1963) was inoculated into monolayers of the Aedes pseudoscutellaris (LSTM-AP-61) cell...
line established by Varma et al. (1974) and the cells incubated at 28 °C. A cytopathic effect (c.p.e.) was observed on the 11th day; some cells became detached and floated free whereas others formed dense clumps. On further passage of the medium on to AP-61 cells, extensive c.p.e. was produced on the third day. By the third passage in mosquito cells, extensive c.p.e. was produced in 24 h.

**Virus production.** Using the methods of Varma et al. (1974, 1975/6) unlabelled virus was obtained by inoculation of AP-61 cells maintained in Leibovitz L-15 medium. Virus labelled with $^3$H-uridine was obtained from infected cells incubated in Leibovitz medium containing the isotope (10 µCi/ml). Virus labelled in the capsid protein was obtained by replacing the medium with methionine-free Eagle’s medium containing $^{35}$S-methionine (10 µCi/ml) 8 h after infection. The virus was titrated in AP-61 cells at 28 °C, using either Falcon flasks, Falcon trays or microtitration trays. The cells were examined after 3 days for c.p.e. and after a further 3 days for plaques. The virus produced plaques using either CM-cellulose or a low gel-point agarose as overlay (De Madrid & Porterfield, 1969; Pudney et al. 1978).

**Purification of virus.** The virus particles were purified by the method described by Brown & Cartwright (1963) for foot-and-mouth disease virus except that 1% SDS was used instead of DOC to resuspend pelleted virus particles. Sucrose gradients (15 to 45%) were prepared in 0.1 M-tris, 0.1 M-NaCl, pH 7.6, and centrifuged for 2 h at 95,000 g. Fractions of 1 ml were collected by puncturing the bottom of the centrifuge tube and the position of the virus particles ascertained by radioactivity measurements.

**Stability of the virus to pH, heat and lipid solvents.** (a) The effect of different pH buffers on the infectivity of virus was determined by mixing 0.5 ml samples of a virus preparation in culture medium containing 2% foetal calf serum (FCS) with 0.5 ml of 0.1 M-acetate buffer to give a pH of 2.7, 3.9, 5.5, 7.6 or 9. After incubating at 23 °C for 20 min, the infectivity of each sample was titrated in AP-61 cell monolayers. (b) The stability of virus infectivity in culture medium containing 2% FCS was determined by incubation at 28 °C. Virus infectivity was determined at selected intervals for a period of 30 days. (c) To determine the stability of virus particles in lipid solvents, 4 vol. of a virus preparation in culture medium containing 2% FCS was thoroughly mixed with 1 vol. of ether in a sealed bottle. After standing for 1 h at 23 °C the ether was removed by evaporation and the infectivity determined.

**Sedimentation coefficient.** A mixture of purified $^{35}$S-methionine labelled Kawino virus particles and $^3$H-uridine labelled poliovirus type 1 particles was centrifuged in a 15 to 45% sucrose gradient for 2 h at 95,000 g and the distribution of the two isotopes determined on the fractionated gradients.

**Buoyant density in CsCl.** Purified Kawino virus particles were layered on top of preformed CsCl gradients (approx. 1.3 to 1.4 g/ml) prepared in 0.1 M-tris, pH 7.6 and pH 9.0 (Rowlands et al. 1971), and centrifuged for 18 h at 75,000 g at 20 °C. Samples from the fractionated gradients were used for measuring the refractive index and radioactivity.

**Virus RNA analysis.** Purified virus particles labelled with $^3$H-uridine were diluted with 2 vol. 0.1 M-acetate, 0.1% SDS at pH 5.0 and extracted twice with water-saturated phenol. The aqueous layer was mixed with approx. 500 µg of BHK cell ribosomal RNA and precipitated twice with 2 vol. ethanol at −20 °C. The precipitate was dissolved in 0.1 M-acetate, 0.1% SDS and centrifuged on a 5 to 25% sucrose gradient for 18 h at 45,000 g. Absorbance at 260 nm and radioactivity were determined on fractionated gradients.

To determine whether Kawino virus RNA contains poly (A), $^3$H-uridine labelled RNA was filtered through a 1.5 x 0.5 cm column of Sepharose poly (U) equilibrated in 0.5 M-NaCl, 0.01 M-tris, pH 7.6, containing 0.5% SDS and the proportion of counts bound was...
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determined. To elute bound RNA 90 % formamide, 10 mM-potassium phosphate, pH 7·5, 10 mM-EDTA and 0·2 % SDS was applied to the column. A control experiment with 3H-uridine labelled EMC virus RNA, which contains poly (A), was also done.

Infectivity of Kawino virus RNA was determined by inoculating monolayers of AP-61 cells after washing them several times to remove all serum.

Virus polypeptide analysis. Purified Kawino and poliovirus particles labelled with 35S and 3H-methionine respectively were disrupted with 1 % SDS, 1 % β-mercaptoethanol and 0·5 M-urea at 100 °C for 2 min and layered on to a 0·6 × 20 cm gel containing 10 % polyacrylamide and 0·3 % (v/v) ethylene diacrylate. Electrophoresis was for 18 h at 8 mA/gel in 0·1 M-sodium phosphate, pH 7·2, containing 0·1 % SDS and 0·1 M-3-mercaptopropionic acid. Slices of 1 mm of the gel were digested in 0·2 N-NaOH for 1 h at 20 °C then mixed with Triton X100/toluene based scintillant (1:2 by volume), shaken for 1 h at 4 °C and counted in a Packard scintillation spectrometer.

RESULTS

Growth of Kawino virus in tissue culture cells

Kawino virus multiplied in the Aedes pseudoscutellaris AP-61, Aedes malayensis AM-60 and Aedes albopictus cell lines at 28 °C (Varma et al. 1974; Singh, 1967) but c.p.e. was evident only in the AP-61 cells. A titre of 10^8·5 cytopathic doses 50 (CPD₅₀) per ml was obtained when the cells were freeze-thawed three times in the maintenance medium 24 h after inoculation. The virus did not grow at 28 °C in the Aedes aegypti, Anopheles stephensi, Rhipicephalus appendiculatus or Xenopus laevis cell lines (Varma & Pudney, 1969; Pudney & Varma, 1971; Varma et al. 1975; Pudney et al. 1973). There was no evidence of growth in three mammalian cell lines tested; there was some maintenance of titre in the pig kidney (PS) cell line although none in BHK 21 and Rhesus monkey kidney (LLC-MK₂). The peak titre of released virus was 10^6·5 CPD₅₀/ml in the A. albopictus cells on day 2, 10^5·9 p.f.u./ml in AP-61 cells on day 3, and 10^3·5 CPD₅₀ in the AM-60 cells on day 3.

Kawino virus that had been passaged three times in mosquito tissue cultures did not kill 1 to 3-day-old Swiss albino suckling mice when 0·02 ml was inoculated intracranially.

Stability of Kawino virus

Kawino virus particle preparations in Leibovitz L-15 medium (Leibovitz, 1963) with 10 % tryptose phosphate broth and 2 % FCS lost no infectivity when left at 28 °C for 30 days. The infectivity was also stable when the preparations were incubated for 20 min at 23 °C in acetate buffers in the pH range 2·7 to 9·0. Furthermore, no infectivity was lost when virus particles were incubated with ether for 1 h at 23 °C.

Physical characterization of the virus particle

The sedimentation coefficient of purified Kawino particles virus in sucrose gradients relative to that of poliovirus particles was determined by centrifuging a mixture of 35S-labelled virus with 3H-poliovirus as described in Methods. From the distribution of radioactivity (Fig. 1a) it is clear that purified Kawino virus particles are homogeneous and by using a value of 160S for the sedimentation coefficient of poliovirus particles (Newman et al. 1973a) the relative value for Kawino virus was estimated to be about 165S.

A sample of the peak fraction of the gradient used for purifying Kawino virus was mixed with 2 % potassium phosphotungstate and examined by electron microscopy. Large numbers of particles were observed, including some which were penetrated by stain and
Fig. 1. (a) Co-sedimentation in a sucrose gradient of $^{35}$S-methionine labelled Kawino virus particles and $^3$H-uridine labelled poliovirus particles. □, $^{35}$S; ○, $^3$H. (b) Equilibrium centrifugation of $^{35}$S-methionine Kawino virus particles in a CsCl gradient in 0.1 M-tris, pH 7.5.

Fig. 2. Electron micrograph of purified Kawino virus particles.
may lack RNA. The virus particles were estimated to have an average diameter of 28 nm (Fig. 2).

The buoyant density of the virus particles was determined by centrifuging 35S-labelled virus particles on preformed CsCl gradients as described in Methods. Symmetrical peaks of radioactivity at a density of 1.33 g/ml were obtained at both pH 7.5 and pH 9.0 (Fig. 1 b). These results show that Kawino virus particles have the same characteristics in CsCl as vertebrate enterovirus particles (Rowlands et al. 1971).

**Analysis of virus RNA**

Ribonucleic acid extracted from 3H-uridine labelled virus particles with phenol–SDS sedimented as a single homogeneous peak at about 35S on sucrose gradients compared with 28S and 18S for the BHK cell ribosomal RNA markers (Fig. 3 a). The relative sedimentation coefficient obtained for the virus RNA corresponds to a mol. wt. of about 2.6 × 10^6 (Spirin, 1964). Pre-treatment with pancreatic ribonuclease at 0.1 μg/ml resulted in hydrolysis to slowly sedimenting molecules (Fig. 3 b). This confirms that the virus nucleic acid is RNA and indicates that it has a single-stranded structure.

The widespread occurrence of poly (A) in the RNA of vertebrate picornaviruses (Frisby et al. 1976) and most eukaryotic cell messenger RNA species (Brawerman, 1974) and the finding that it was absent from the genome of Nodamura virus (Newman & Brown, 1976), led us to determine whether the Kawino virus genome contained poly (A). Whereas 80 % of the EMC virus RNA bound to Sepharose poly (U) and was eluted by the addition of 90 % formamide, only about 11 % of the Kawino virus RNA was bound (Table 1). These results suggest that the Kawino virus genome is unlike those of the vertebrate picornaviruses. It is
Table 1. Affinity chromatography of EMC and Kawino virus RNA on Sepharose poly (U)

<table>
<thead>
<tr>
<th></th>
<th>EMC virus RNA</th>
<th>Kawino virus RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ct/min %</td>
<td>ct/min %</td>
</tr>
<tr>
<td>Unbound</td>
<td>1582 20</td>
<td>4608 89</td>
</tr>
<tr>
<td>Bound</td>
<td>6397 80</td>
<td>582 11</td>
</tr>
</tbody>
</table>

Fig. 4. Co-electrophoresis in a polyacrylamide gel of the polypeptides from ³⁵S-methionine labelled Kawino virus particles and ³H-methionine labelled poliovirus particles. ——, ³⁵S; —,—, ³H.

possible, however, that the Kawino virus genome possesses a poly (A) tract that does not bind to Sepharose poly (U) because of its length or because it is masked by the configuration of the RNA.

The infectivity titre of a preparation of Kawino virus RNA in AP-61 cells was $10^{6.5}$ CPD₅₀/ml compared with $10^{6.6}$ CPD₅₀/ml for the preparation of virus particles from which it was extracted. Treatment of the RNA with 0.1 μg/ml pancreatic ribonuclease for 5 min at room temperature before adding to the cell monolayer destroyed its infectivity.

Analysis of virus proteins

When ³⁵S-methionine labelled Kawino virion protein was electrophoresed in 10% polyacrylamide gels it separated into four peaks of radioactivity. The mol. wt. of the polypeptides were estimated by co-electrophoresis with ³H-methionine labelled poliovirus polypeptides (Fig. 4). Using mol. wt. values of 31, 27, 25 and $8 \times 10^3$ for the poliovirus polypeptides (Abraham & Cooper, 1975), the mol. wt. of the Kawino virus polypeptides were estimated to be 33, 30, 27 and about $7 \times 10^3$. In some virus preparations a minor component with a mol. wt. of $36 \times 10^3$ was also detected and this may correspond to the protein called VPO that is found in many picornavirus particles (Rueckert, 1971).
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Table 2. Comparison of the physico-chemical properties of Kawino virus and poliovirus

<table>
<thead>
<tr>
<th>Property</th>
<th>Kawino virus</th>
<th>Poliovirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus Diameter (nm)</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td>Sedimentation coefficient (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.5</td>
<td>c. 165</td>
<td>160</td>
</tr>
<tr>
<td>pH 9.0</td>
<td>1.33</td>
<td>1.34</td>
</tr>
<tr>
<td>Density in CsCl (g/ml) at:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.5</td>
<td>1.3</td>
<td>1.34</td>
</tr>
<tr>
<td>pH 9.0</td>
<td>1.33</td>
<td>1.34</td>
</tr>
<tr>
<td>RNA Sedimentation coefficient (S)</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Contains poly (A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1</td>
<td>33</td>
<td>31</td>
</tr>
<tr>
<td>VP2</td>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td>VP3 mol. wt. ( \times 10^{-3} )</td>
<td>27</td>
<td>25</td>
</tr>
<tr>
<td>VP4</td>
<td>c. 7</td>
<td>8</td>
</tr>
<tr>
<td>Stability at pH 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stable</td>
<td>Stable</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

The physico-chemical properties of Kawino virus particles which are summarized and compared with those of poliovirus particles in Table 2, suggest that the mosquito virus should be included in the enterovirus sub-group of the Picornaviridae. However, one striking difference between Kawino virus and the picornaviruses is the absence of a poly (A) tract from the infective RNA of the mosquito virus. The possession of a poly (A) tract has been regarded by Spector & Baltimore (1974) as being necessary for the infectivity of poliovirus RNA and the infectivity of EMC virus RNA has been correlated with the length of the poly (A) tract (Goldstein et al. 1976; Hruby & Roberts, 1977). Whether virus RNA does not need a poly (A) tract to infect invertebrates is not known and it would be interesting to determine whether Drosophila C virus, another entero-like virus from which infective RNA can be extracted (Jousset et al. 1977), also lacks a poly (A) tract.

It is now becoming apparent that there are entero-like viruses infecting a wide range of invertebrates and that these are widely distributed throughout the world. Preliminary studies using immunodiffusion tests have shown that Kawino virus is unrelated to Drosophila C virus (L. Bailey, personal communication) and Gonometta virus (our unpublished observations), two invertebrate viruses possessing the physico-chemical properties of the vertebrate enteroviruses. However, a survey of the serological relationships between the invertebrate and vertebrate enteroviruses would be valuable, particularly in view of the observation by Longworth et al. (1973) that Gonometta virus, a virus isolated only in East Africa that infects Gonometta podocarpi (Lepidoptera; Lasiocampidae), was precipitated by sera obtained from several vertebrate species in Britain, an area where the virus has not been found.

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