A nonoccluded reovirus of the olive fly, *Dacus oleae*

Maria Anagnou-Veroniki,1 Jean-Claude Veyrunes,2 Georges Kuhl2 and Max Bergoin2

1 NAGREF, Benaki Phytopathological Institute, 14561, Kiphissia (Athens), Greece
2 Unité de Virologie Moléculaire, Station de Recherches de Pathologie Comparée, 30380 St Christol-lez-Alès, France

We have isolated paraspherical viral particles, 60 nm in diameter, from adults of the olive fly (*Dacus oleae*) collected in Greece. The virus actively replicated in midgut epithelial cells and in advanced infections virions accumulated in microvilli. They were released in the gut lumen and were very abundant in fly faeces. The virions exhibited the salient features of reoviruses, with an external shell and an internal core with a tubular subunit protruding at each vertex of the icosahedron. The viral genome consisted of ten segments of double-stranded RNA totalling 23.4 kbp. Based on its overall properties, this virus can be considered as a nonoccluded insect reovirus.

The olive fly, *Dacus oleae*, is one of the major pests of olive trees all around the Mediterranean basin. Damage caused by the larvae, which feed on olive fruits, is severe and economically important (Arambourg, 1986). The most commonly used way to control this pest is by spreading organophosphate compounds mixed with attractants to kill adults. Several alternative methods of integrated control including male sterilization (Tzanakakis, 1967), the use of pheromone baits (Haniotakis et al., 1987; Broumas et al., 1990) or natural endoparasites (Arambourg, 1986) have also been developed. Identification of new pathogens could provide further means for efficient control of this fly. In a survey for viruses in natural populations of *D. oleae*, we identified three types of nonoccluded isometric viral particles approximately 60, 40 and 30 nm in diameter in crude extracts of adults examined by electron microscopy (Anagnou-Veroniki et al., 1984, 1991). The largest of these was isolated from flies collected in the island of Evia (Greece) and was tentatively identified as a nonoccluded reovirus on the basis of its size and morphology. We have further characterized this virus and the data reported here confirm that it shares the main structural and genomic features of the members of the family *Reoviridae*.

The virus was multiplied by per os infection of virus-free *D. oleae* flies (Marathon strain). Adult flies were maintained at 26–28 °C, 60–70% relative humidity and an 18 h per day photoperiod and were fed on artificial liquid diet (Arambourg, 1986). To identify the site of virus replication, tissue fragments were collected from infected flies, fixed in 2% (v/v) glutaraldehyde in 0–1 M-phosphate buffer (pH 7.4), post-fixed in 2% aqueous osmium tetroxide, dehydrated through a graded series of acetone, and embedded in epoxy 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and were observed using a Zeiss EM 10 CR transmission electron microscope.

Examination of different tissues (hypoderm, nervous chain, muscle, tracheoblasts, foregut and midgut) revealed that infection was apparently confined to midgut epithelial cells. In heavily infected cells, electron dense material with a granular structure occupied large cytoplasmic areas. The presence of viral particles in the process of assembly identified these areas as viroplasms (Fig. 1a). After maturation, viral particles accumulated at the periphery of viroplasms to form para-crystalline arrays. Hexagonal outlines of virions in these aggregates generated honeycomb images (Fig. 1b).

Virions were also abundant in the distal regions of midgut epithelial cells near the microvilli (Fig. 1c). As a result of their accumulation in microvilli, these became hypertrophied (Fig. 1d) and apparently detached from the cells. Free virions were also observed in the gut lumen (Fig. 1c). This process of virus release, which mimics exocytosis or the liberation of virions by disruption of heavily infected cells, explains our observations of large quantities of viral particles in negatively stained preparations of faeces from adult flies (data not shown).

To further investigate the fine structure and the genome of the viral particles, highly purified viral suspensions were prepared. Infected flies were macerated in 0.05 M-phosphate buffer, pH 7.4, and the homogenate was clarified at 2500 g for 10 min. The virions were sedimented at 75 000 g for 1 h. The virus pellet was dispersed in the same buffer plus 0.03% sodium deoxycholate by gentle sonication and the virus layered on top of a 10–50% (w/w) linear sucrose gradient. After centrifugation for 1 h at 150 000 g in a Beckman SW41 rotor, analysis of the gradient by recording the absorbance at 254 nm using an Isco gradient collector apparatus showed a major peak in the middle of the gradient column preceded by a shoulder (data not shown). Examination by electron mi-
Fig. 1. Reovirus infection of *Dacus oleae* midgut epithelial cells. (a) Viral particles in the process of assembly in electron dense virogenic stroma (VS). Virions are accumulating at the periphery to form paracrystalline arrays. Bar represents 0.5 µm. (b) Detail showing the honeycomb structure resulting from the ordered arrangement of icosahedral particles. Bar represents 0.5 µm. (c) Apical region of a cell showing accumulation of virions near the microvilli. The arrow indicates a virus free in the gut lumen. Bar represents 0.5 µm. (d) Hypertrophied microvilli filled with virions. Bar represents 0.6 µm.
Nonoccluded reovirus of *D. oleae*.

**Fig. 2.** Purified virus particles and cores negatively stained with PTA. (a) Intact virions showing the row of subunits forming the outer shell and internal core. Bar represents 0.1 µm. (b) Degraded particles showing the structure of the viral core. Hollow projections typical of cypovirus cores are clearly visible at the vertices of the icosahedron. Bar represents 0.1 µm.

The nucleic acid type contained in the virus was first determined by colorimetric methods. A purified virus suspension reacted positively with orcinol (Mejbaum, 1939) and negatively with diphenylamine (Giles & Myers, 1965), thus demonstrating the ribonucleic acid nature of the viral genome. The small amount of virus sample did not allow us to estimate the RNA content of the virion. We further characterized the viral genome by PAGE. Viral suspensions were incubated for 2 h at 37 °C with 0.1 mg/ml proteinase K, 1.8% (w/w) Sarkosyl in TEN (0.01 M-Tris, 0.004 M-EDTA, 0.1 M-NaCl, pH 8.0) buffer and the RNA was analysed by PAGE using 5% stacking, 7% running gels. Gels were stained with 0.5 µg/ml ethidium bromide and photographed under UV light. We analysed, under the same conditions, RNA extracted from *Bombyx mori* cypovirus (*Bm cypovirus*), which served as marker. To this end, a suspension of *Bm* cypovirus polyhedra was prepared from heavily infected silkworm midguts and virus particles were extracted from purified polyhedra as described by Payne & Rivers (1976).

The electrophoretic profile of the viral RNA was similar to that of *Bm* cypovirus thus providing evidence that the *D. oleae* virus contains a segmented double-stranded (ds)RNA genome (Fig. 3). Eight bands were observed but the intensity of both the second and third (bands B and C, Fig. 3) strongly suggested that each corresponded to a doublet of segments with very...
The virus isolated from adult olive flies shared similar sizes. Thus, the viral genome very likely consists of 10 segments of dsRNA. A similar situation was observed with the viral genome of Bm cytopivirus where the second band (B) could not be resolved into its two RNA segments (Fig. 3). The size of the D. oleae viral genomic fragments was calculated by comparison with the known size of Bm cytopivirus RNA segments (Fuji-Kawata et al., 1970). The total viral genome has a size of 23.4 kbp for D. oleae reovirus compared with 22.65 kbp for Bm cytopivirus. By comparison with the size classes of orthoreovirus genomic segments (Ramig et al., 1977), the RNA segments of the D. oleae reovirus genome could be assigned to class L for segments 1 to 3, to class M for segments 4 to 6 and to class S for segments 7 to 10.

Both by its morphology and its segmented dsRNA genome, the virus isolated from adult olive flies shared the main features of members of the family Reoviridae (Murphy et al., 1995). As with insect reoviruses of the genus Cytopivirus, the infection seemed to be confined to midgut epithelial cells and the viral core exhibited the typical hollow spikes located at the vertices of the internal icosahedral capsid (Payne & Mertens, 1983). However, D. oleae reovirus exhibits the clearly defined outer shell characteristic of orthoreoviruses and, unlike cytopviruses, no occlusion body was formed. In this respect, D. oleae reovirus resembles the nonoccluded reoviruses previously reported to infect Musca domestica (Moussa, 1978), Drosophila melanogaster cell lines and adult flies (Haars et al., 1980; Plus et al., 1981a), and the trypetid fly Ceratitis capitata (Plus et al., 1981a, b). More recently, virus particles with similar morphology were observed in stocks of D. oleae collected in different areas of Northern Greece (Manousis et al., 1986; Manousis & Moore, 1987). This confirmed our previous report of the wide distribution of the virus in both continental and island populations of D. oleae in Greece (Anagnou-Veroniki et al., 1984). Of significant epidemiological interest is the observation of large quantities of virions in faeces of adult flies. This excretion very likely contributes to the spreading of the virus both horizontally and vertically by contamination of the larval food and eggs during oviposition in olives. The impact of the virus in natural conditions is still unknown but we have demonstrated that in laboratory conditions the virus is pathogenic both per os and by inoculation (Anagnou-Veroniki et al., 1984).

Although the sum of the 10 segments of D. oleae reovirus and B. mori cytopivirus genomes was similar, the electrophoretic patterns of the two genomes were clearly distinct. The D. oleae reovirus genomic profile also differed from those published for four strains of the F reovirus of D. melanogaster (Plus et al., 1981a) but no clear conclusion could be drawn from this comparison since electrophoretic conditions were different.

Based on the morphology of the virions, the lack of polyhedra and possibly on genomic profiles, the nonoccluded reoviruses of insects should be classified in a genus apart from cytopviruses since the term nonoccluded cytopivirus is misleading.

References


Received 2 May 1996; Accepted 23 August 1996