W2 Virus Infection of the Crustacean *Carcinus mediterraneus*: a Reovirus Disease

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SUMMARY

Most of the viruses described in marine invertebrates have been related to known virus families only on the basis of ultrastructural properties. Recently a viral agent was isolated and studied in the Mediterranean shore crab *Carcinus mediterraneus*. This agent, which was 65 to 70 nm in diameter, developed in the cytoplasm of connective tissue cells of *C. mediterraneus* and produced unusual viral structures, 'rosettes', consisting of an empty sphere bounded by arrangements of viral particles. The capsid consisted of two protein shells. After purification, full virions exhibited a density of 1.34 g/ml in CsCl. The nucleic acid composition of virions was estimated at about 22% and was shown to be a dsRNA with at least nine segments in four different size classes. The capsid contained six polypeptides with \( M_r \) of \( 120 \times 10^3 \), \( 94 \times 10^3 \), \( 76 \times 10^3 \), \( 44 \times 10^3 \), \( 32 \times 10^3 \) and \( 24 \times 10^3 \), as determined by SDS–PAGE. From its biological, ultrastructural and physicochemical properties, we propose that this virus should be classified as a new member of the family Reoviridae.

INTRODUCTION

Reports on viral diseases of marine invertebrates have increased in number in the past 20 years, largely due to the need for pathological studies following the development of marine culture. Numerous viruses have been described and, principally on the basis of morphological and ultrastructural observations, classified in known virus families (Couch, 1981). Only one has been sufficiently characterized to be accepted by the International Committee on Taxonomy of Viruses as a member of the family Baculoviridae (Matthews, 1982). This virus was the agent of the nuclear polyhedrosis of the pink shrimp *Penaeus duorarum*, which has been named BP for 'Baculovirus penaei' (Couch, 1974a, b). This characterization was principally supported by a demonstration of the circular structure of the nucleic acid, extracted directly from inclusion bodies (Summers, 1977).

To date, several different viruses isolated from Crustacea have been reported (Johnson, 1983, 1984), some of which have been partially characterized such as S virus, P virus (Bonami, 1980) and PC 84 virus (Mari & Bonami, 1988). A new viral agent which is pathogenic for Crustacea was isolated from Channel coast specimens of the Atlantic shore crab *Carcinus maenas* (J. R. Bonami & B. J. Hill, unpublished observations). This agent, which we suggested was closely related to members of the family Reoviridae, was designated W virus. More recently, a viral agent was observed in diseased Mediterranean shore crabs *C. mediterraneus* (Mari & Bonami, 1986) from the Mediterranean coasts. Based on ultrastructural similarities with W virus, it was designated W2 virus. In this report we consider pathological, ultrastructural, biophysical and biochemical properties of this agent.

METHODS

Animals. Crabs were caught in the Prevost lagoon and along the Mediterranean coast near Montpellier (France). They were maintained in aerated sea water-filled tanks (501) and fed with fresh mussels twice a week.
Virus. The W2 virus was originally isolated from one naturally infected *C. mediterraneus* found in a batch of 30 crabs. Later, it was again isolated from other animals from the same biotope.

Experimental transmission of the disease. Crabs isolated from their natural environment were maintained in quarantine for 2 weeks in the laboratory before being used for experiments. Lethargic animals were discarded; only the most vigorous crabs were selected for transmission experiments; there was no sex distinction. The selected crabs were injected with 0.2 ml of infectious material in a leg joint of the fifth pair of pereiopods. The inoculum was either an homogenate of infected tissue in TN buffer (0.02 M-Tris–HCl, 0.5 M-NaCl, pH 7–2), or a purified virus suspension.

Electron microscopy. Tissue samples were fixed in glutaraldehyde–osmium tetroxide as described by Perkins (1969) and embedded in Epon. Ultrathin sections were stained according to the method of Reynolds (1963) and examined in an Hitachi HU 11B electron microscope operating at 75 kV. Semithin sections were stained with toluidine blue. Purified viral suspensions or tissue homogenates were negatively stained with 2% sodium phosphotungstate (PTA) at pH 7.

Virus purification. Hepatopancreas, gut and gills of infected crabs were homogenized in TN buffer using a Potter tissue blender. After centrifugation to clarify it (15 min at 2500 g), the suspension was centrifuged for 1 h at 50000 g. Pellets were resuspended in TN buffer, mixed with an equal volume of Freon (1:1,2 trichloro-2,2,1 trifluoroethane), shaken vigorously and centrifuged for a few min at 2500 g. The upper aqueous phase was removed and re-extracted two or three times with Freon. The final extract was layered onto a linear gradient of sucrose (30 to 50% w/w) in TN buffer and centrifuged for 2.5 h at 130000 g. Fractions were separated using a Büchler Auto Densi-Flow equipped with a u.v. absorbance monitor (Isco UA5) operating at 254 nm.

Isopycnic centrifugation. Purified virus fractions obtained from the sucrose gradient were diluted in TN buffer and centrifuged for 1 h at 130000 g. Resuspended pellets were layered on a preformed linear CsCl gradient (20 to 48% w/w) and centrifuged for 15 h at 130000 g. For analysis, fractions of full virions obtained from the CsCl gradient were diluted in an appropriate buffer, either SSC (0.15 M-NaCl, 0.015 M-sodium citrate, pH 7) or 0.016 M-Tris–HCl, centrifuged for 1 h at 130000 g and resuspended in one of the previously described buffers.

Spectrometric analysis. The nature of nucleic acid was determined by the diphenylamine reaction (Giles & Myers, 1965) and the orcinol reaction according to Mejbaum (1939). Protein concentration of virus preparations was measured using the Folin reaction (Lowry et al., 1951) as modified by Hartree (1972). Absorption spectra of purified suspensions or extracted nucleic acid were measured with a double beam Perkin-Elmer 550 spectrophotometer equipped with an external recorder. Melting profiles of extracted nucleic acid in SSC buffer were obtained using a Perkin-Elmer temperature programmer operating at 1 °C/min; the absorption was recorded at 260 nm.

Nucleic acid extraction. Purified virus suspensions were treated with 0.5 M-sarkosyl and with pre-incubated proteinase K (50 μg/ml) for 30 min at 37 °C. The viral RNA was precipitated with cold ethanol after three or four phenol extractions and resuspended in SSC buffer.

Effect of formaldehyde. Neutralized formaldehyde was added to a viral suspension to give a final concentration of 1.8% v/v. The absorption spectrum was recorded immediately, at different times during a 24 h period, and finally after heating samples at 100 °C for 10 min.

Agarose gel electrophoresis. Viral RNA was fractionated by agarose gel electrophoresis using the submarine method in an LKB Maxiphor tank. The electrophoresis buffer of Peacock & Dingman (1968) was modified with 0.1% SDS. Samples of W2 virions were processed in dissociating medium (1% SDS, 1% mercaptoethanol and 6 M-urea) and incubated for 1 h at 37 °C. Viral RNA or gel-purified genome segments were heated at 37 °C for 10 min before electrophoresis. Bacteriophage λ DNA treated with endonuclease HindIII and bacteriophage φX174 DNA cut with *Hae*III were used as Mr markers. Bands were observed on a u.v. transilluminator after staining with 0.001% ethidium bromide in 20 mM-ammonium acetate, pH 7.7. Fragments of the viral genome were recovered from low melting temperature agarose gels by melting the agarose, phenol extraction and cold alcohol precipitation (Sealey & Southern, 1982).

**RESULTS**

Experimental disease and histopathology

Disease was experimentally reproduced by the injection of a partially purified W2 virus suspension previously prepared from naturally infected animals. Symptoms of infection
A reovirus disease of Carcinus mediterraneus included absence of aggressiveness, increasing weakness and lack of appetite. The duration of the experimental disease was about 7 to 20 days, ending with the death of the crabs. W2 virus was detected by electron microscopy of infected animals as early as the 5th day after injection. From the 7th day all moribund animals contained W2 virions.

The connective tissue of many organs (hepatopancreas, digestive tract, gills, hematopoietic organs) of W2-infected animals showed severe damage. The most obvious lesions were observed in the connective tissue surrounding the tubules of the hepatopancreas (Fig. 1), although all cellular types were progressively destroyed. Connective tissue was replaced by isolated cells, degenerative cells and debris which did not exhibit any organization. Inside these necrotic areas, various nodules appeared which consisted of debris surrounded by aggregated haemocytes. Epithelial cells of the digestive tract, gills and hepatopancreas did not seem to be affected by the disease.

**Cytopathology**

Using electron microscopy it was possible to study the extensive cellular degeneration in tissues where lesions were previously observed. Cells and debris derived from connective tissue and blood cells replaced normal structures. Inclusions and organelles were modified, e.g. mitochondria lost their internal organization so that cristae were not observed. Vacuolization of some cells was noticeable, but abnormal structures also occurred scattered within the cytoplasm which was filled with numerous free ribosomes (Fig. 2a). The perinuclear cisternae were enlarged in some places and the amount of dense chromatin decreased. The abnormal formations observed in the cytoplasm were dense areas of variable size, sometimes associated with tubular structures 25 to 30 nm thick (Fig. 2b).

Another very unusual structure was present which consisted of electron-dense rings within the cytoplasm of many cells. These rings, or ‘rosettes’, occurred due to the clustering together of five to seven paraspherical elements, 55 to 60 nm in diameter (Fig. 2a, b, c). The ‘rosettes’ resembled non-enveloped virus particles. Together with these formations, it was sometimes possible to recognize paraspherical elements scattered freely in the cytoplasm; some could be observed around or within the dense inclusions associated with the tubular structures. Numerous free ribosomes were seen around the ‘rosettes’.

Virus-like particles of a near-uniform shape and size were also observed in the haemolymph and were sometimes aggregated with cellular debris into slightly electron-dense amorphous inclusions. Virions, released from disrupted cells, did not show the characteristic ‘rosette’ associations, but outer and inner capsid structures were more noticeable (Fig. 3).

Electron microscopy confirmed that connective tissue was the only tissue affected by this disease. Neither viral particles nor lesions were observed in epithelial tissues.

**Ultrastructure of virions in tissues**

Virus particles were most frequently associated in ‘rosettes’ of 70 to 130 nm diameter. These features seemed to consist of an empty sphere bounded by particles. This was supported by the observation that the most frequent form of ‘rosette’ comprised six or seven virions. Some forms, however, were composed of only three, four or five virions in which case a distinct central region was not observed, suggesting that the section was not cut through the centre of the sphere. The ultrastructure of the particles contained within the ‘rosettes’ was difficult to interpret because not all of the particles were at the same level in the section and electron-dense areas often surrounded the ‘rosettes’ (Fig. 2c).

In contrast, when virions were isolated, either in an infected cell or in the extracellular space, their ultrastructure was more obvious. They were paraspherical, non-enveloped, 57 to 65 nm in diameter, composed of a 15 to 20 nm thick outer layer (capsid) and an electron-dense centre 30 to 35 nm in diameter (Fig. 3).

**Ultrastructure of purified virions**

The morphology of virions was easier to interpret after negative staining of purified viral suspensions. These were obtained from infected crab organs where the occurrence of W2 virus
had been previously confirmed by PTA negative staining of a crude macerate of hepatopancreas. After centrifugation on a sucrose gradient, two very close bands were observed. After gradient fractionation with an Auto Densi-Flow these two bands usually gave only one peak. After this had been concentrated in TN buffer the suspension contained empty and full particles of W2 virus. By CsCl linear gradient centrifugation it was possible to separate the two bands without any contamination (Fig. 4). The upper band was composed of empty particles and occasional tubule-like structures whereas the lower band contained full particles.
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Negative staining revealed paraspherical particles, 65 to 70 nm in diameter (Fig. 5). Subunits, 8 to 9 nm in diameter, were distinguished on the surface of the particles, the dense centres of which (5 nm in diameter) suggested a prismatic structure or an empty tubule. This subdivision into capsomers was confirmed by a crenellated appearance around the empty particles that had been penetrated by the PTA. These empty particles exhibited a hexagonal electron-dense centre, 50 to 55 nm in diameter, surrounded by a capsid 8 to 10 nm thick. Some capsids of empty particles seemed to consist of two layers of the same thickness separated by a dark space.

The occurrence of a double capsid was also more noticeable on full particles when the outer shell had become damaged (Fig. 6a, b, c, d, e). The outer shell, 4 to 5 nm thick, showed structures with a surface made of capsomers. Cores, released after the breakdown of the outer shell, resembled small viral particles exhibiting a superficial subunit organization. Some of these cores were paraspherical in shape, others were hexagonal, about 55 to 66 nm in diameter (Fig. 6e).

Older viral suspensions revealed the presence of paracrystalline areas near the virions (Fig. 7). These areas consisted of small empty prismatic units, identical in size to the capsomer-like structures of virus-like particles. They were probably a rearrangement of the capsid subunits derived from damaged virions. Some tubules were observed among the suspensions of empty virions. They were 70 nm in diameter and variable in length (Fig. 8). They consisted of small subunits, 10 nm in diameter. Some tubules exhibited a superficial striated appearance, 10 nm wide, perpendicular to the long axis.

Morphogenesis

W2 virus morphogenesis occurred in the cytoplasm, the only place where normal virions were associated in ‘rosettes’. During the early stages of infection, virions appeared within viroplasms which were located in several regions of the cytoplasm. These viroplasms were electron-dense granular regions with some clear filamentous areas. Very dark granular areas were observed mainly around virus ‘rosettes’.

At a later stage of virogenesis, the cytoplasm of infected cells appeared more homogeneous; it contained a large number of ribosomes. In the vicinity of the viroplasms, some less dense elements with irregular limits were observed (Fig. 9). Some of these contained tubular
formations, 25 nm in diameter, which also appeared alone in the cytoplasm of infected cells. Free virions, not included in 'rosettes' were also observed scattered around the electron-dense areas (Fig. 9).

**Physicochemical properties**

The densities of the two bands obtained after isopycnic CsCl gradient centrifugation, were 1.275 ± 0.005 g/ml for empty particles, and 1.340 ± 0.005 g/ml for full particles (Fig. 4).

The absorption spectrum of full particles was characteristic of nucleoproteins (max. 257 nm; min. 244 nm). The $A_{260}/A_{280}$ was 1.57, corresponding to a proportion of nucleic acid of about 15% according to Layne (1957).

When virus particles were tested with diphenylamine or orcinol, the diphenylamine reaction (Giles & Myers, 1965) was negative but the orcinol reaction (Mejbaum, 1939) was positive, indicating that the viral nucleic acid was RNA. After determination of protein content (Hartree, 1972), the proportion of nucleic acid was estimated at about 22 ± 2%.

Addition of formaldehyde did not cause any hyperchromicity during a 24 h period. After heating, hyperchromicity was noted, indicating a dsRNA structure in particles. The absorption spectrum of extracted dsRNA was typical with a max. at 257 and a min. at 232 nm. Ratios of absorbance between 260 and 280 and between 260 and 230 nm were 2:1 and 1:73 respectively. The thermal denaturation recorded at 260 nm showed an increase in absorbance between 90 and 100 °C corresponding to an hyperchromicity of 31% which was a property of a double-stranded nucleic acid. The melting temperature ($T_m$) was 96 °C in 1 × SSC.

The polypeptides of full particles were separated by SDS-PAGE. After staining with Coomassie Brilliant Blue, four major polypeptides were observed of $M_r$ 120 × 10$^3$, 94 × 10$^3$, 32 × 10$^3$ and 24 × 10$^3$. In addition to these four polypeptides, a more sensitive silver staining (Merril et al., 1982) revealed two other minor components of 76 × 10$^3$ and 44 × 10$^3$ (Fig. 10). Depending on the viral suspensions used, these two minor polypeptides gave more or less dense bands suggesting that they were elements probably from the outer shell.

Submarine electrophoresis in agarose gel (0.9 or 1%) revealed at least nine RNA bands (Fig. 11). The total $M_r$ for the genome using a λ DNA HindIII/φX174 DNA HaeIII digest as markers was estimated at 13.49 × 10$^6$, with an $M_r$ for each band of 3.06 × 10$^6$, 1.88 × 10$^6$, 1.69 × 10$^6$, 1.57 × 10$^6$, 1.51 × 10$^6$, 1.10 × 10$^6$, 0.92 × 10$^6$, 0.87 × 10$^6$ and 0.83 × 10$^6$. Electrophoresis of extracted viral nucleic acid in agarose gel (0.8, 0.9 and 1%) in Peacock & Dingman buffer (1968) gave the same electrophoretic profile as the viral suspensions, i.e. nine segments. The best definition of these nine bands was obtained with 1% agarose gel. To study the possible occurrence of double bands, segment 1, segments 2, 3 and 5, segment 6 and segments 7, 8 and 9 were studied separately. After a first electrophoresis in 0.6% agarose, the pieces of gel corresponding to these four groups were cut out and the nucleic acid was extracted. Then the four samples were analysed using electrophoresis at several gel concentrations (0.5 to 1%), voltages and times of migration. No additional bands were observed.

**DISCUSSION**

The signs of disease observed during the W2 virus infection of *C. mediterraneus* was similar to those described for other viral infections of this crab (Mari, 1987). W2 virus develops in the connective tissue of different organs, but lesions do not show unique properties which would
allow easy diagnosis of the disease; indeed similar lesions (i.e. connective cell necrosis) have been described in other viral diseases of crabs (Bonami, 1980; Johnson, 1977). Lesions of W2 virus disease can be distinguished from those of P virus disease of *Macropipus depurator* (Bonami, 1977) and reo-like virus disease of *Callinectes sapidus* (Johnson & Bodammer, 1975; Johnson, 1977) by the occurrence during these two latter diseases of fuschinophilic formations which correspond to crystalline arrays of virions.

By its size, structure and cytoplasmic development, the W2 virus appears to be closely related to the family Reoviridae (Matthews, 1982). Nevertheless, the formation of ‘rosette’ structures is very unusual in this family. Such ‘rosette’ structures have been reported for the reo-like virus of the gills of *C. mediterraneus* (Bonami, 1980) and the W virus of *C. maenas* (J. R. Bonami & B. J. Hill, unpublished observations).

Tubular formations, similar in size to those observed in W2 virus-infected cells, have been
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reported in P virus, W virus and RLV infections. Similar but smaller formations have also been described in the case of the reo-like viruses of the gills of *C. mediterraneus*. However, an original aspect of infections with W2 virus, and which has not been reported in previous viral infections of crabs, is the presence of electron-dense material sometimes containing tubular formations and virions. These did not exhibit an organized structure and did not seem to be similar to the paracrystalline inclusion bodies of the type described in insect cytoplasmic polyhedrosis infections (Bird, 1965; Vago & Bergoin, 1968; Payne & Mertens, 1983).

On negative staining of purified particles, a double-shelled capsid was observed; such a feature, characteristic of several but not all genera of the Reoviridae (Matthews, 1982), has also been observed in W virions (J. R. Bonami & B. J. Hill, unpublished observations) and P virions (Bonami, 1977, 1980) of Crustacea. The capsid structure of the W2 virus strongly resembles the capsomer arrangement of some members of the Reovirus (Smith *et al.*, 1969; Wood, 1973; Joklik, 1983), Rotavirus (Palmer *et al.*, 1977; Esparza & Gil, 1978; Holmes, 1983) and Orbivirus genera (Wood, 1973). However, we have not observed structures resembling the spikes described in cytoplasmic polyhedrosis virus and on the cores of Reovirus (Hosaka & Aizawa, 1964; Joklik, 1974; Payne & Harrap, 1977; Joklik, 1983) or the A and B spikes of Fijivirus (Boccardo *et al.*, 1980).

Virus morphogenesis occurs in small dense areas scattered in the cytoplasm; we interpret these as viroplasms. The virogenesis reported in the case of P virus of *M. depurator* (Bonami, 1980) was similar to this, but unlike the morphogenesis observed in cytoplasmic virosis of the scorpion *Buthus occitanus* (Morel, 1975, 1979), Fijivirus (Boccardo *et al.*, 1980) or cytoplasmic polyhedrosis virus (Xeros, 1956; Bird, 1965; Payne & Mertens, 1983).

Whatever the extent of cell infection, full virions were generally assembled in ‘rosettes’. To our knowledge, such structures have never been reported in the family Reoviridae. W2 virus infection, like that of P virus (Bonami, 1980) and RLV (Johnson & Bodammer, 1975; Johnson, 1977), appears to lead to the production of tubular structures as well as virions. Similar structures have also been described in the course of infections by some members of the Reoviridae (Wood, 1973; Boccardo *et al.*, 1980).

The density of W2 virions (1.39 g/ml) and the proportion of nucleic acid (22%) are consistent with values generally obtained for members of the Reoviridae (Matthews, 1982). The dsRNA genome confirms the close relationship with this family. The viral RNA $T_m$ (96 °C) obtained in 1 × SSC appears higher than values generally obtained for members of Reoviridae (Gorman *et al.*, 1983), but is consistent with these values if we consider that they have been obtained using a buffer of 10-fold lower ionic strength (0.1 × SSC).

As for Reovirus, W2 polypeptides are distributed into three size classes. We assume that the two minor polypeptides are in close association with proteins of the external shell because they were not always present in our different virus preparations. They have an $M_r$ of $73 \times 10^3$ and $44 \times 10^3$ respectively, quite comparable with the $\mu 2$ (or $\mu lc$) and $\sigma 1$ polypeptides known to be constitutive proteins of the external shell of Reovirus (Joklik, 1972, 1985).

The W2 dsRNA genome is multisegmented like that of other members of the Reoviridae (Matthews, 1982) although only nine segments were resolved from W2 instead of the more usual 10 to 12. The possible existence of one or more double bands made of two segments of the same $M_r$ cannot be discounted; therefore more accurate detection techniques such as electrophoresis in 7.5 or 10% polyacrylamide should be considered (Payne & Mertens, 1983). The presence of nine segments does not constitute a fundamental difference from established members of the Reoviridae; because some members of this family (as yet unclassified within a genus), possess a genome containing only eight segments (Matthews, 1982). Another difference from established members of the Reoviridae is the distribution of W2 dsRNA segments in four size classes instead of three. However a distribution in four size classes has been reported for some human rotavirus strains (Lourenco *et al.*, 1982; Georges *et al.*, 1983; Arista *et al.*, 1983).

From its biological, ultrastructural and physicochemical properties, the W2 virus does not correspond to any of the defined genera of the Reoviridae, but most of its characteristics are similar to those reported for this family. We therefore propose that the W2 virus of the marine crustacean *C. mediterraneus* should be considered a new member of the family Reoviridae.
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