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A reolike virus of the Mediterranean shore crab *Carcinus mediterraneus*

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ABSTRACT: During studies on viral diseases of the Mediterranean shore crab *Carcinus mediterraneus*, a new viral agent has been found. It localizes in epithelial cells of hepatopancreatic tubules, especially inside B-cells. Virions, paraspherical in shape, non-enveloped, 70 to 75 nm in diameter, form large cytoplasmic viral areas. Generally, groups of virions do not exhibit an ordered arrangement; however some paracrystalline clusters have been observed. By its characteristics, size, shape, absence of envelope and cytoplasmic development, this agent can be closely related to the Reoviridae.

INTRODUCTION

Over the last 20 yr, knowledge of pathogens occurring in marine decapod crustaceans has been extended to viral agents. Several viruses or virus-like particles have been described in crabs and shrimps in the course of investigations either on undetermined mortalities or during ultrastructural studies (Bonami 1980, 1987, Couch 1981, Johnson 1983, 1984, Lightner 1983).

Some of these crustacean species are of economic importance, whereas others, such as *Carcinus mediterraneus*, have been chosen as biological models. This crab is very common on Mediterranean coasts, in Languedoc lagoons, and can conveniently be maintained in laboratories.

Several viral agents have been described in *Carcinus mediterraneus* (Pappalardo & Bonami 1979, Mari & Bonami 1986), and the occurrence of viral infections showing a complex etiology appears to be a very interesting aspect. Concomitant replication of several viruses is generally unusual, but occurs frequently in marine crustaceans, particularly in crabs (Bonami 1980, Johnson 1984). In the course of our investigations on these viral associations in *C. mediterraneus*, a new viral agent was evidenced in hepatopancreatic epithelium and named RC84. The purpose of this paper is to describe its ultrastructural and cytopathological properties.

MATERIAL AND METHODS

Animals. Crabs *Carcinus mediterraneus* Czs. were caught in the Prevost lagoon near Montpellier (France) and maintained in 50 l tanks supplied with aerated and recirculated seawater. They were fed with fresh mussels twice a week.

Electron microscopy. Hepatopancreas, digestive tract and gills were fixed in 2% glutaraldehyde in cacodylate buffer 0.2M, NaCl 0.4M, followed by 1% osmium acid in the same buffer. After dehydration, tissues were embedded in Epon. Ultrathin sections were prepared using a LKB Ultrotome V, contrasted according to Reynolds (1963) and observed in a Hitachi HU11B electron microscope operating at 75 kV.

Examination of crab tissues for the presence of viruses was also carried out by direct observation of tissue homogenates after negative staining with 2% sodium phosphotungstate (PTA) at pH 7.

Histology. Semithin sections were stained with Toluidine blue.

RESULTS

During a 4 yr period (1983 to 1986), our observations indicated that RC84 virus occurred rarely in wild populations of *Carcinus mediterraneus*. Infected crabs

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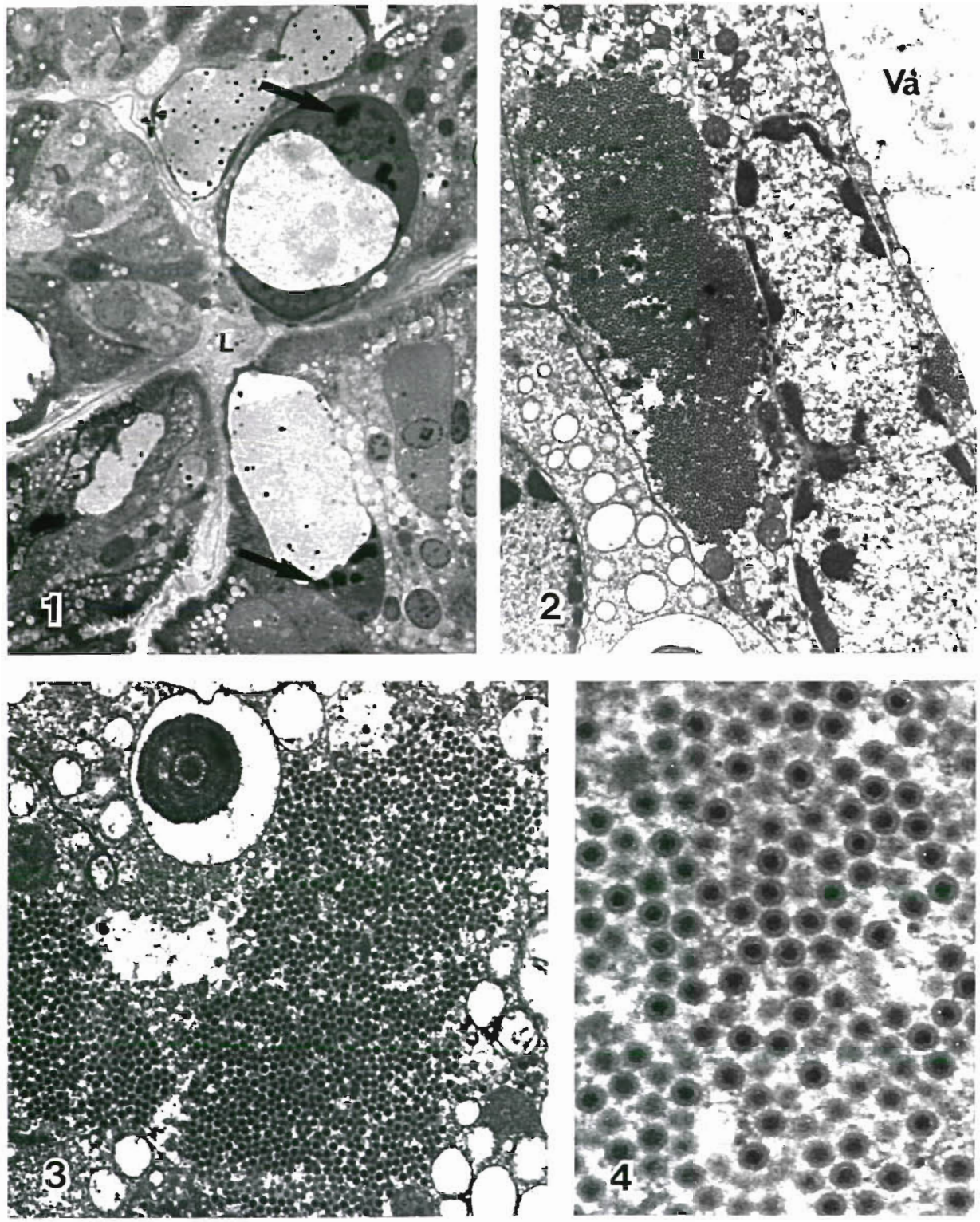

Fig. 1 to 4. *Carcinus mediterraneus*. Fig. 1. Semithin section of infected hepatopancreatic epithelium. Dark-blue stained inclusions (arrow) are present in the cytoplasm of B-cells. L: lumen of the tubule. Toluidine blue; × 450. Fig. 2. Viral area in cytoplasm of an infected B-cell. Va: vacuole. EM, × 7500. Fig. 3. Viral area showing numerous ribosomes and small empty vacuoles. Note the presence of a calcium granule. EM × 17 000. Fig. 4. Detail of virions in cytoplasm. EM × 58 500

showed an atypical clinical symptomatology, i. e. signs of disease were like some of those seen in other viral infections, such as lethargy, loss of appetite and weakness. Indeed, RC84 virus was observed in crabs showing complex virosis (simultaneous infections by 2 or 3 viruses).

HISTOLOGY

In semithin sections, abnormal structures were observed in B-cells of the digestive epithelium of the hepatopancreas (the cellular types of hepatopancreatic epithelium are named here according to terminology defined by Jacobs [1928] which is generally accepted for crabs: Johnson 1980). In normal B-cells, the nucleus is forced into a basal position by a large vacuole, and the cytoplasm in *Carcinus mediterraneus* does not show many granules or special inclusions (Pappalardo 1981). In contrast, in diseased tissues, dark-blue stained inclusions were present in the cytoplasm around the large vacuole (Fig. 1). In infected tissues, B-cells appeared to be more numerous than in healthy tissues. B-cells containing abnormal inclusions were scattered among apparently normal B-cells.

Inclusions were of a small size around the vacuole and larger in the basal area of the cell. Rarely, they were observed near calcium spherules, which are not a characteristic of B-cells in *Carcinus mediterraneus*, but are very numerous in R-cells (Pappalardo 1981). E and F-cells did not exhibit any abnormal inclusions. Except for the presence of the virus, there were no abnormalities in the epithelial cells, even in the proximal zone of the hepatopancreatic tubules where B-cells were numerous.

Electron microscopy

Ultrathin sections

Electron microscopy revealed that the dark-blue stained inclusions previously observed in cytoplasm of B-cells were formed by accumulation of viral particles (Fig. 2). These viral areas, scattered in the cytoplasm around the central vacuole, were variable in shape and size. Some of them reached a length of 8 µm. Numerous small empty vacuoles were observed at the periphery of the viral areas (Fig. 3). Some filamentous and diffuse material, often associated with a large number of ribosomes, made very electron-dense areas in the cytoplasm. Frequently such structures were present inside or around a viral area, suggesting a viroplasm. The nuclear structure seemed to be unaltered.

Virions (Fig. 4), 70 to 75 nm in diameter, consisted of an external layer, 15 nm thick, corresponding to the capsid, and of an electron-dense core, 40 nm in diameter. Generally, virions were clustered, but with clear zones containing no particles inside the viral area.

Occasionally, virions were disposed in straight lines, suggesting a paracrystalline arrangement, with a 60 nm period (Fig. 5); we have to emphasize that this distance is incompatible with the virion diameter; moreover this structure appears to be 3-dimensional, due to the fact that straight lines were not layered on the same level.

The RC84 virus is specific for B-cells. However in case of heavy infections, some particles were observed in the vicinity of calcium spherules in some R-like cells; RC84 virions were never observed in E and F-cells.

Virions were noted in the lumen of hepatopancreatic tubules, probably released by disruption of the host cells. They were associated with cell debris or isolated as small clusters (Fig. 6). Some particles were entrapped between apical microvilli of epithelial cells (Fig. 7). These particles could be the source of the spreading of the disease, among host cells, and/or in crab populations by release to the aquatic environment.

The RC84 virus did not occur in any other organ of infected crabs.

Ultrastructure of isolated particles

This agent was studied in negatively stained hepatopancreatic homogenates from infected crabs which were previously examined for RC84 virus infection by study of ultrathin sections.

Particles were paraspherical in shape, 85 to 95 nm in diameter. Their aspect and size were quite different from known viruses of *Carcinus mediterraneus* (Mari & Bonami 1986). They often appeared in small bundles of 3 to 10 particles. In each group, virions were more or less penetrated by PTA, allowing us to make out viral components (Fig. 8a, b). Some virions were electron-lucent, without noticeable superficial ornamentation, and were interpreted as being intact mature virions. Other particles showed a superficial layer surrounding an inner structure, paraspherical in shape, 65 to 70 nm in diameter. More damaged particles, penetrated by PTA, appeared as 2 shells surrounding an electron-dense center, 50 nm in diameter. These 2 concentric shells were separated by a space of variable thickness. The internal shell, 6 to 6.5 nm thick, exhibited small projections or spikes. The external shell, 8.5 to 9.5 nm thick, did not possess any ornamentation. It seemed to be fragile and gaps were observed in PTA-penetrated particles; sometimes a single external shell seemed to surround 2 cores.

DISCUSSION AND CONCLUSION

The RC84 virus, paraspherical in shape, 70 to 75 nm in diameter, non-enveloped, forms large viral areas in the cytoplasm of digestive epithelial cells. These cytoplasmic formations do not show evidence of specific arrangements, but in some cases paracrystalline arrays have been observed.

This virus differs from other described viruses in crabs by its strict location in digestive epithelium. All the other known reolike viruses of crabs grow in connective tissue and hemocytes, except for the reolike virus of *Carcinus mediterraneus* gills (Bonami 1977, 1980), which is described as infecting solely the gill epithelium.

A second difference from other reolike viruses of crabs is the general absence of ordered arrangements of virions in the cytoplasm; the P virus of *Macropipus depurator* (Bonami et al. 1976) and RLV of *Callinectes sapidus* (Johnson & Bodammer 1975, Johnson 1977) typically exhibit mainly large paracrystalline arrays,

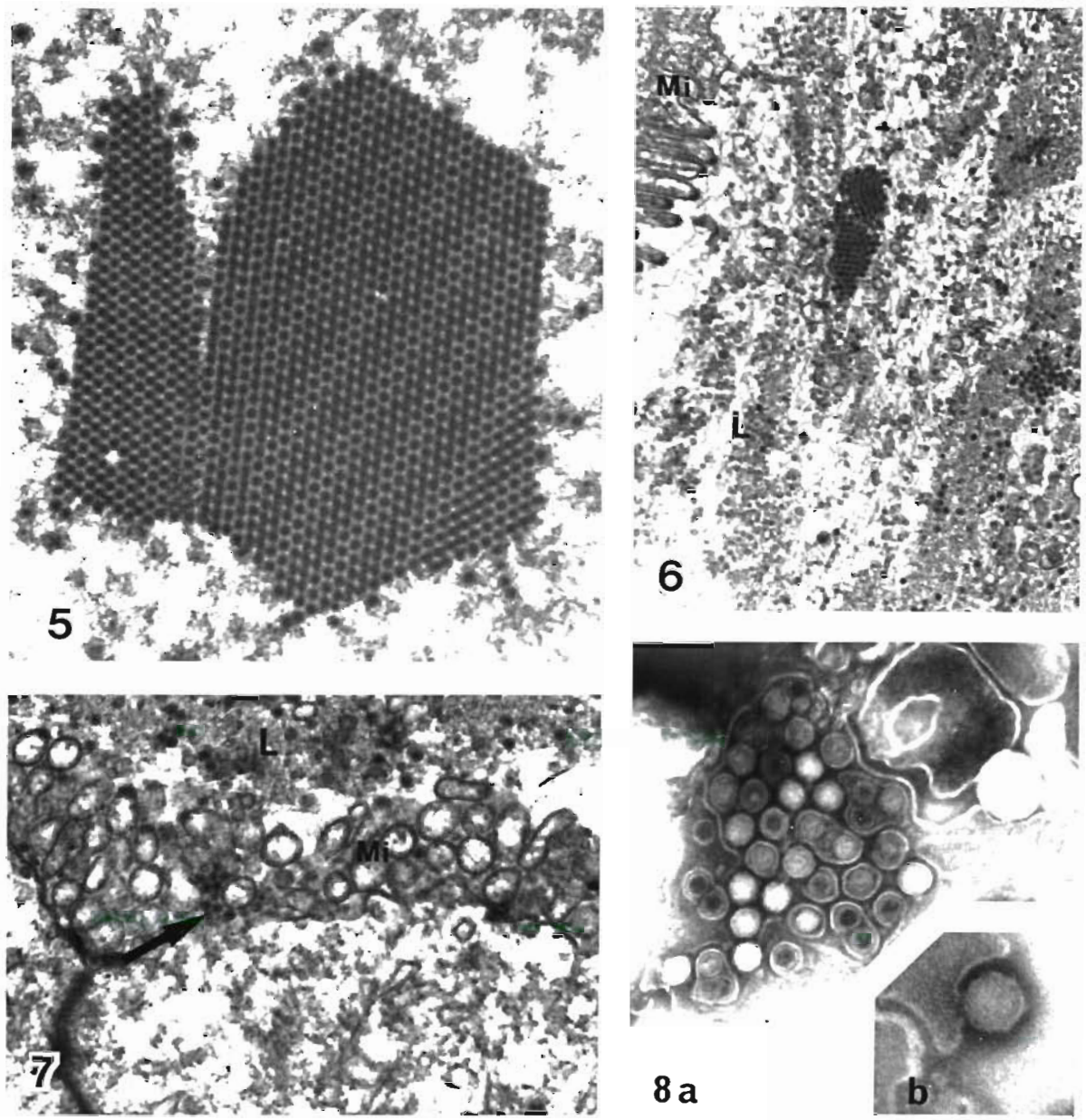

Fig. 5 to 8. *Carcinus mediterraneus*. Fig. 5. Paracrystalline array in cytoplasm of infected B-cell. EM, × 26 000. Fig. 6. Cluster of virions released into the lumen (L) of a hepatopancreatic tubule. Mi: microvilli. EM, × 12 800. Fig. 7. Group of RC84 virions (arrow) entrapped between microvilli of epithelial cells limiting the lumen (L) of the tubule. EM × 21 300. Fig. 8. Negatively stained RC84 virions. (a) Different stages of virus degradation, × 52 000. (b) Undamaged mature viral particle, × 110 000

whereas W of *Carcinus maenas* (Bonami & Hill unpubl.), W2 of *C. mediterraneus* (Mari & Bonami 1986) and the reolike virus of *C. mediterraneus* gills (Bonami 1977, 1980) produce rosette arrangements in sections. All these reolike viruses are associated with membranous structures which have never been observed in RC84-infected cells.

Another difference bears on the size of virions which are larger in RC84 (70 to 75 nm) than in all the other reolike viruses of crabs (about 55 to 65 nm).

At the level of marine crustacean virology, the RC84 virus appears to be a new agent; only one other closely morphologically related virus is known; it was found in the shrimp *Penaeus japonicus* (Tsing & Bonami 1986, 1987). As in RC84, the virus of *P. japonicus* is located in epithelial cells of hepatopancreas and does not form an ordered arrangement. However these 2 viruses are different in size: 70 to 75 nm and ca 60 nm respectively.

A feature of RC84 virus is its high specificity to only one cell type of the hepatopancreatic epithelium; while *Penaeus japonicus* virus develops inside R-cells, RC84 virus develops inside B-cells and, in case of heavy infections, probably in R-cells. This high specificity is not found in the other *Carcinus mediterraneus* viruses that develop in epithelial cells of hepatopancreas. In fact, the τ (Tau) baculovirus infects nuclei of all cell types of hepatopancreatic epithelium (Pappalardo & Bonami 1979, Pappalardo 1981, Pappalardo et al. 1986).

As previously emphasized (Bonami 1980, Johnson 1983, 1984), multiple viral infections occur very often in crabs. Even though, in the same individual, different tissues can be simultaneously infected, multiple infections of the digestive epithelium have never been observed. Indeed, even though τ virus and RC84 virus can each be found associated with concomitant viral infections of connective tissue, they have never been observed developing together in the same individual.

An ultrastructural feature of isolated RC84 virions is the occurrence of a double shell constituting the capsid. Such a structure, characteristic of Reoviridae (Matthews 1982), has been reported in Crustacea for the P virus of *Macropipus depurator* (Bonami et al. 1976) and the W2 virus of *Carcinus mediterraneus* (Mari 1987). However, in RC84, these 2 shells often appear separated from one another, unlike the classical picture of negatively stained reovirions (Wood 1973, Palmer et al. 1977, Boccardo et al. 1980). The presence of a single external shell surrounding 2 or more cores can be interpreted either as an artifact due to the proximity of damaged particles or to a rearrangement of proteinic subunits of the fragile external shell.

Although the nature and structure of the agent's nucleic acid were not analyzed, by its characteristics this virus can be closely related to the Reoviridae (Matthews 1982).

Experimental transmission studies of the virus are in progress to define its pathogenicity and to produce virions for biochemical investigations. Isolated RC84 virus possesses a fragile external shell which is easily lost during purification procedures and when this happens, the virions look like W2 virus in negatively stained preparations. Special attention must be paid to prevent any confusion between the 2 viruses.

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