Herpesviruses associated with mortalities among hatchery-reared larval Pacific oysters *Crassostrea gigas*

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ABSTRACT: Larvae of hatchery-reared Pacific oysters *Crassostrea gigas* Thunberg ceased feeding 3 to 4 d after spawning, and 60 to 100 % mortalities occurred after 7 to 11 d. Up to 25 % of moribund larvae had putative phagocyte precursors and fibroblasts with enlarged nuclei of abnormal shape and chromatin pattern, which contained ovoid to hexagonal, non-enveloped, 97 ± 4 nm diameter capsids and nucleocapsids of a herpes-like virus. Nucleocapsids became enveloped and de-enveloped as they passed through the nuclear membranes. Some became enveloped in intranuclear sacs containing tubular elements. Naked cytoplasmic nucleocapsids became enveloped by budding into vesicles or Golgi cisternae, and probably acquired a tegument from large dense granular masses near Golgi complexes. Extracellular enveloped virions usually had a tegument, and were 131 ± 9 nm in diameter. Extracellular naked nucleocapsids were thought to derive from lysed cells. Non-enveloped ovoid to hexagonal 86 ± 12 nm virions, thought to be degraded herpesvirions, were abundant in inter-digitating processes of putative phagocytes in >50 % of moribund larvae. Comparison with groups within the Herpesviridae was made, and similarities to cytomegaloviruses noted. Elevated temperature and crowding may increase susceptibility of oyster larvae to herpes-like virus infection.

INTRODUCTION

Pacific or Japanese oysters *Crassostrea gigas* Thunberg are increasingly being cultured in many countries, from stocks ultimately originating from Japan. Whereas introductions into North America, Europe and Australia (Tasmania) were by human agency, *C. gigas* was first reported in New Zealand in 1971 (Dinamani 1971), and was probably accidentally introduced on Japanese vessels in the late 1960's (Smith et al. 1986). In many cases movement of *C. gigas* has occurred before adequate disease studies could be undertaken, and therefore international movement of *C. gigas* stocks may have resulted in the spread of pathogens of *C. gigas*. The international movement of spat poses a continuing risk of disease introduction, until pathogens in such spat have been identified.

Three viral diseases have been reported from *Crassostrea gigas*. All 3 were caused by iridoviruses, 2 of which occurred in adult *C. gigas* (syn. *C. angulata*) in France (Comps 1988). The third iridovirus caused Oyster Velar Virus Disease (OVVD) in hatchery-reared

larval *C. gigas* in the northwest United States (Elston & Wilkinson 1985). Four viruses have been reported from *Crassostrea virginica* in the northeast United States, a herpes-type virus in oysters held at elevated temperatures (Farley et al. 1972), a papovavirus and a retrovirus (Farley 1978), and a reo-like virus in juvenile oysters (Meyer 1979). Herpesviruses have also been observed in cells with Cowdry type A inclusions in about 4 % of oysters *Ostrea edulis* in North Wales during summer (Alderman 1980, pers. comm.).

In mid-summer (February) 1991 mass (60 to 100 %) mortalities occurred in larval *Crassostrea gigas* in a hatchery at Mahurangi, 40 km north of Auckland, New Zealand, and moribund larvae were examined under the electron microscope. This paper reports the presence and appearance of herpesviruses in those larvae.

MATERIALS AND METHODS

Ten consecutive batches of larvae from locally collected wild oysters *Crassostrea gigas* in spawning con-

dition were held in tanks of filtered (10 μm) seawater at 24.0 to 25.5°C, with water changed every 2 to 3 d. Mortality was estimated from the proportion of larvae in the water column (healthy), or alive but unable to swim normally (moribund), to those that were dead. Wild larvae from plankton taken near the hatchery, and moribund and healthy hatchery-reared larvae sampled daily 7 to 10 d after spawning, were fixed in 2.5% glutaraldehyde in 0.22 μm filtered seawater for 1 to 2 h, washed twice in filtered seawater, and centrifuged to form a pellet; the seawater was removed and replaced by 2% molten (50°C) agar. The pellet in agar was transferred to a slide and cut to small pieces in 0.1 M cacodylate buffer, post-fixed in 1% OsO_4 in 0.1 M cacodylate for 1 h, de-calcified in 5% EDTA (ethylenediaminetetra-acetic acid) in 0.1 M cacodylate for 30 to 45 min, washed in 0.1 M cacodylate, dehydrated in an ethanol series (50 to 100%), washed twice for 10 min in propylene oxide, infiltrated for 1 h in 50/50 propylene oxide/araldite resin, embedded in 100% resin overnight, and cured for 48 h at 60°C. Ultrathin sections were cut and stained in 5% uranyl acetate in 50% ethanol for 10 min and 5% lead citrate in boiled distilled water for 5 min, and examined under a Philips 201 transmission electron microscope.

RESULTS

Course of disease

Larval growth slowed, and in most cases ceased, 3 to 4 d after spawning, coinciding with reduction in feeding and swimming activity. Significant (> 60%) mortality occurred by Day 7, with 100% mortality by Day 11 in most batches. Moribund larvae swam weakly in circles with their vela noticeably less extended than in healthy larvae. Dead larvae measured 75 to 80 μm and were all at the straight-hinge (D) stage. Larvae surviving until 12 d went on to settle normally. Healthy larvae reached 120 μm and entered the umbo stage at 7 d, with settlement at 21 to 28 d when larvae reached 300 to 350 μm .

Ultrastructural observations

In ca 10% of Day-7 and ca 25% of Day-9 moribund hatchery larvae, enlarged cells with hypertrophied nuclei, marginated chromatin and intranuclear inclu-

sions (Fig. 1) contained capsids and nucleocapsids (Fig. 2). Capsids concentrated near ovoid or ring-shaped fine granular masses were empty, or contained a loose central aggregation of fine granular material similar to that of the nuclear inclusion (Figs. 1, 3 & 4). Nucleoid formation or acquisition was not observed, even though nucleocapsids that lacked a tegument and envelope were scattered throughout the nucleus (Fig. 2). However, membrane-bound intranuclear sac-like inclusions near the nuclear periphery contained enveloped virions, and membranous tubular (25 nm diameter) or vesicular structures (Figs. 2 & 5). Enveloped virions were also observed in the perinuclear cisterna (Fig. 6), but most nucleocapsids appeared to enter the cytoplasm without an envelope. Reduplication of the nuclear membrane was not observed. A few large dense granular bodies, lacking a bounding membrane, and closely associated Golgi bodies of normal appearance were observed in each infected cell. Nucleocapsids around the dense bodies (Fig. 7) and nearby Golgi (Fig. 8) were enveloped by budding into vesicles or cisternae. Cytoplasmic nucleocapsids were also seen near large vesicles or cisternae containing a fine reticulated matrix, into which they passed (Fig. 9) and became enveloped (Fig. 10). Whether these large vesicles/cisternae originated from Golgi or the nuclear membrane was unclear. Only naked cytoplasmic nucleocapsids were observed in the cytoplasm of myocytes containing non-striated fibrils (Fig. 11), which were probably adductor muscle cells, but enveloped tegumented single virions were observed in cytoplasmic vesicles in other cells (Fig. 12). Extracellular virions were usually enveloped and had spike-like protrusions on the surface (Fig. 13) or occasionally had a tail (Fig. 14). Degenerating and lysing infected nuclei were frequently observed (Fig. 15). The locations of different stages occurring in cellular compartments are shown in Table 1.

Mature virions consisted of a hexagonal or toroidal core that was 12 nm inside diameter, 66 nm across (Fig. 13), and 43 nm in depth, when viewed longitudinally (Fig. 16). The toroid was enclosed by an elongated hexagonal to ovoid capsid, 97 ± 4 nm ($n = 50$) in diameter, with fine fibrils spanning the lucent space from core to capsid (Figs. 9 & 16). Enveloped virions, 131 ± 9 nm ($n = 50$) across, were bounded by a unit membrane and the tegument appeared electron-dense (Fig. 13). Other than fibroblasts, the infected cell types could not be identified with certainty, but nucleocap-

Figs. 1 to 4. Herpes-like virus infecting *Crassostrea gigas* larvae. Fig. 1. Section showing enlarged cells (arrows) with hypertrophied nuclei that contain marginated chromatin and an intranuclear inclusion (arrowhead); $\times 5950$. Fig. 2. Part of an enlarged cell showing intranuclear capsids (c), nucleocapsids (nc) and an intranuclear sac (is), extracellular tegumented virions (ev) and partially enveloped nucleocapsid (arrow); $\times 47\,960$. Fig. 3. Ring-shaped fine granular intranuclear inclusion; $\times 30\,000$. Fig. 4. Intranuclear fine granular inclusion bordered by capsids containing similar granular material; $\times 50\,000$.

Table 1. Occurrence of different stages of replication of oyster herpes-like virus in different cellular and extracellular compartments of *Crassostrea gigas* larvae

Compartment	Capsids	Nucleocapsids	Enveloping virions	Enveloped virions	Enveloped and tegumented virions
Nucleus	+	+			
Intranuclear sac				+	
Perinuclear space				+	
Cytoplasm		+	+		
Dense bodies		+	+	+	+
Golgi apparatus		+	+	+	+
Cytoplasmic vesicles				+	+
Extracellular		+		+	+

sids 87 to 89 nm in diameter occurred in inter-digitating cells (Fig. 17) that might have been phagocyte (haemocyte) precursors (R. Elston pers. comm.).

Putative degenerating capsids and nucleocapsids were observed in inter-digitating cells of < 50 % of wild larvae and > 50 % of moribund hatchery larvae. They were hexagonal to ovoid in shape, 86 ± 12 nm ($n = 50$) in diameter, with a granular core (Fig. 18).

DISCUSSION

The virions described resemble herpesviruses in size, the toroidal core, possession of an envelope with surface spikes, amorphous tegument, nucleocapsid assembly in the nucleus and occurrence of enveloped virions at the nuclear membrane or in vesicular cytoplasmic spaces or cisternae (Roizman et al. 1981, Murphy & Kingsbury 1990, Roizman 1990). In addition, the fibrils running from the inner surface of the capsid to the torus are similar to the arrangement in herpesvirions (Furlong et al. 1972, Roizman 1990). From the occurrence of different stages in different cellular compartments (Table 1), it seems nucleocapsids are formed in the nucleus, pass through the inner nuclear membrane to become enveloped in the perinuclear space, and lose the envelope on passing through the outer membrane into the cytoplasm. Intranuclear sacs may be invaginations of the inner nuclear membrane into the nucleus. In the cytoplasm, nucleocapsids congregate at dense body/Golgi complexes, where they bud

into Golgi cisternae to become enveloped with or without an amorphous dense tegument that probably derives from the dense body. Enveloped virions in vesicles are released at the cell surface. Extracellular naked nucleocapsids may derive from lysed infected cells.

The herpesviruses reported here show similarities to some members of the Alphaherpesvirinae (the simplexviruses), such as channel catfish virus, in the appearance of the fine granular intranuclear inclusions (Wolf & Darlington 1971). The oyster viruses differ from simplexviruses in lack of reduplication of the nuclear membrane (Smith & de Harven 1973) or modification of the inner nuclear membrane before envelopment (Campadelli-Fiume et al. 1991). Also, simplexviruses are enveloped on budding through the inner nuclear membrane and remain enveloped as they pass down a labyrinth of endoplasmic reticulum, vesicles or cisternae and out of the cell. Thus naked nucleocapsids are seldom observed in the cytoplasm, except for mutant strains (Campadelli-Fiume et al. 1991). Simplexviruses have intranuclear sacs termed tegusomes (Roffman et al. 1990), in which the tegument is formed. The sacs lack tubular elements and often contain cytoplasmic structures (Wolf & Darlington 1971), and are therefore unlike those observed here. Dense bodies are not involved in transit of virions through the cytoplasm.

This oyster virus more closely resembles the Betaherpesvirinae [the cytomegaloviruses (CMV)] in the enlargement of infected cells and their nuclei (Roizman et al. 1981), the presence of intranuclear sacs containing

Figs. 5 to 10. Herpes-like virus infecting *Crassostrea gigas* larvae. Fig. 5. Intranuclear sac containing enveloped virion (ev) and tubular (t) and vesicular (v) structures; $\times 120\,000$. Fig. 6. Enveloped virion (ev) in the perinuclear space near fine granular inclusion ($\times 36\,000$), and detail of enveloped virion (inset; $\times 96\,000$). Fig. 7. Dense granular cytoplasmic body surrounded by nucleocapsids, some of which are becoming enveloped by cytoplasmic vesicles or cisternae (arrow); $\times 44\,330$. Fig. 8. Golgi profile showing dense granular material (arrows) and cisternae containing granular material around naked nucleocapsids; $\times 56\,250$. Fig. 9. Nucleocapsids budding into vesicle or cisterna containing reticulated material. Note nearby naked nucleocapsids and extracellular enveloped virions with a dense tegument; $\times 90\,060$. Fig. 10. Enveloped virions within vesicle or cisterna, and unenveloped nucleocapsids free in the cytoplasm; $\times 154\,620$.

Figs. 11 to 18. Herpes-like virus infecting *Crassostrea gigas* larvae. Fig. 11. Myocyte cytoplasm containing unenveloped nucleocapsids; $\times 52\,780$. Fig. 12. Enveloped tegumented virion within a cytoplasmic vesicle; $\times 143\,930$. Fig. 13. Extracellular enveloped tegumented virion showing the toroidal core and surface spikes (arrows); $\times 173\,900$. Fig. 14. Extracellular enveloped virion with a tail containing amorphous tegument; $\times 121\,890$. Fig. 15. Lysed nucleus showing intranuclear nucleocapsids (arrowheads); $\times 17\,630$. Fig. 16. Enveloped virion showing a longitudinal section of the toroidal core and fine filaments passing from it to the inside of the capsid; $\times 210\,690$. Fig. 17. Inter-digitating portion of cell containing nucleocapsids with a toroidal core; $\times 116\,000$. Fig. 18. Inter-digitating portion of cell showing nucleocapsids containing granular pleomorphic cores; $\times 110\,400$.

tubular elements (Tumilowicz & Powell 1990), the temporary envelopment and de-envelopment as nucleocapsids pass through the nuclear membrane (Severi et al. 1979), and their association with dense bodies

and Golgi in the cytoplasm (Severi et al. 1988). The ovoid ring of nuclear granular material (Fig. 4) is similar to that reported in human cytomegalovirus infection (Tumilowicz & Powell 1990). If cessation of feed-

ing by *Crassostrea gigas* larvae at 3 to 4 d indicates onset of infection, and most deaths occur at 9 to 10 d, replication may occur over ca 6 d, as in CMV (Smith & de Harven 1973, Roizman et al. 1981), although several replication cycles may occur over this period. In *C. virginica*, the free intranuclear tubular structures in herpesvirus infection (Fig. 3 in Farley et al. 1972) closely resemble intranuclear tubules in CMV infection (Fig. 2 in Fong et al. 1979). However, the large characteristic intranuclear inclusions of CMV (Fons & Albrecht 1986) have not been observed in this study, and the multiple dense bodies of CMV pass with virions out of the cell (Fong et al. 1979). The peripheral vesicles around dense bodies and 'black holes' reported from CMV infection (Severi et al. 1988) were also not observed here. Despite this, the general process of CMV replication outlined by Severi et al. (1988: Fig. 10, routes A/A1, B and C) is similar to larval oyster herpes-like virus replication.

The replication of Gammaherpesvirinae (saimirine viruses) also involves intranuclear sacs with tubular elements and many dense fibrillar bodies (Tralka et al. 1977, Luetzeler et al. 1979), but intranuclear tubular lamella-particle complexes (Tralka et al. 1977), intranuclear electron-dense granules and occurrence of nucleocapsids within dense bodies (Luetzeler et al. 1979) were not observed here. More importantly, the specificity of gammaherpesviruses for T and B lymphocytes (Roizman et al. 1988) makes invertebrate infection unlikely.

The larval oyster herpesvirus may appear, on the basis of transmission electron microscopy (TEM), to be more similar to the Betaherpesvirinae than to the other 2 herpesvirus sub-families, but such similarities cannot be taken to indicate affinities. Details of replication are variable within sub-families (Dargan 1986) and vary with cell type infected (Tralka et al. 1977, Dargan 1986), strain of virus (Campadelli-Fiume et al. 1991) and time after infection (Severi et al. 1988), and the variables concerned need to be tested before a general pattern can be determined. Herpesviruses of aquatic organisms are poorly defined at present, and further studies may well establish further sub-families within the Herpesviridae.

There have been several reports of herpesviruses or herpes-like viruses from aquatic animals (Buchanan & Richards 1982, Hedrick & Sano 1989, Hedrick et al. 1990), some of which cause typical Cowdry type A inclusions (Buchanan & Richards 1982, Sano et al. 1985, Hedrick & Sano 1989), including herpes-type viruses in oysters (Farley et al. 1972, Alderman 1980). The nucleocapsids described here are similar in dimension to those of other aquatic herpesviruses, including those observed in *Ostrea edulis* by Alderman (1980, pers. comm.), but at 97 ± 4 nm they are larger than the 70

to 90 nm of nucleocapsids described from *Crassostrea virginica* by Farley et al. (1972). However, virion dimensions depend on the method of measurement and specimen preparation, and the herpesviruses of oysters may be conspecific.

The method by which larvae become infected is unknown, but Meyers (1981) reported Cowdry type A inclusions in the mantle epithelial cells of an adult *Crassostrea virginica* in spring from Long Island, USA. The mantle epithelium is an ideal site for shedding viruses, and it may be that eggs or larvae are infected by virions shed by the parent oyster around spawning. However, the presence of virions in these Cowdry type A inclusions needs to be confirmed by TEM.

In this study growth and development of larvae were retarded, presumably because of cessation in feeding, and mortality was high. Virus-associated mortalities were confined to between 7 and 11 d after spawning, beyond which larvae appeared refractory to the virus. Herpesviruses of marine organisms may cause disease under crowded, stressed conditions at elevated temperatures (Buchanan & Richards 1982), as here, although such conditions favour pathogens in general, and do not apply to all aquatic herpesviruses (Skinner & Mizell 1972, Hedrick et al. 1990). However, the occurrence of herpesviruses and mortalities in summer in *Ostrea edulis* in North Wales (Alderman 1980), the presence of herpesviruses in oysters held at elevated temperatures in Maine (Farley et al. 1972), and the occurrence of mortalities in mid-summer in this study suggest oyster herpesviruses may only be a problem at elevated temperatures, particularly under crowded hatchery conditions. If this virus has affinities with the Betaherpesvirinae, it is likely to have narrow host specificity and may be specific to ostreids.

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