Studies on *Herpesvirus scophthalmi* infection of turbot *Scophthalmus maximus* (L.) ultrastructural observations

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Abstract. Recent heavy mortalities amongst 0+ group turbot at a fish farm were found to be associated with a herpes-type viral infection of the epithelia of the skin and gills. The morphology of the virus is described with ultrastructural observations on its morphogenesis and release from infected cells.

Introduction

Herpes-like viruses have been described from channel catfish, *Ictalurus punctatus* (Rafinesque) by Wolf & Darlington (1971); from carp, *Cyprinus carpio* L. by Schubert (1966) and from fresh water salmonids, by Wolf & Taylor (1975), Wolf, Herman, Darlington & Taylor (1975) and Wolf, Sano & Kimura (1975). In a preliminary note (Buchanan, Richards, Sommerville & Madeley 1978) we reported the presence of a herpes-like virus in skin and gill lesions of farmed turbot *Scophthalmus maximus* (L.) and that similar lesions are also found less frequently in wild fish. Richards & Buchanan (1978) described the histopathology of this disease as seen in the light microscope and this paper describes the electron microscopic findings, including stages in the replication of the virus.

Materials and methods

Details of the infected and control fish used in this study have already been published (Richards & Buchanan 1978).

Preparation of specimens for thin section electron microscopy

Wet preparations of skin scrapings were examined by phase contrast light microscopy for the presence of the characteristic giant cells and those showing a high proportion were fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer overnight. After thorough rinsing in buffer solution they were post-fixed in 1% osmium tetroxide in 0.05 M sym-collidine buffer for 1 h at 4°C followed by dehydration in a graded series.

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of alcohols and embedding in epoxy resin (EMscope Ltd.). Sections were cut on a
Reichert OMU 3 ultramicrotome, stained with uranyl acetate and lead citrate or with
1% potassium permanganate and examined in a JEOL 100C electron microscope.
Similar preparations were made from control turbot from which giant cells were
absent. The blocks were trimmed on an LKB Pyramitome and 0.2 μm sections were
cut and stained with toluidine blue. These were examined under phase and differen-
tial interference phase optics to locate the giant cells and serial 500 Å sections were
taken for examination by electron microscopy.

Negative staining techniques

Scrapings of skin from infected and normal fish were suspended in Eagle’s tissue
culture medium. Approximately 1 ml of skin scrapings and medium were transferred
to a ten Broek homogenizer and 2 ml of distilled water were added. After homogeniza-
tion the resultant slurry was clarified and the supernatant concentrated by further
centrifugation at 100,000 g for 30 min. The pellet was resuspended in 2 drops of
EM diluent (0.1% Bacitracin) and mixed with an equal volume of negative stain.
One drop of this mixture was applied to a carbon-Formvar coated grid and the surplus
drawn off with the edge of a filter paper. The grid was then examined in a Philips 301
electron microscope at an accelerating voltage of 80 kV or in a JEOL 100C at an
accelerating voltage of 100 kV.

The negative stains used were 3% potassium phosphotungstate (PTA) and 4%
ammonium molybdate, both at pH 7. Initially, a variety of treatments were used to
remove mucus from the skin scrapes but these were later found not to be necessary.

Results

The cytopathic effects of viral infection

Examination of 0.2 μm sections of epoxy resin embedded skin scrapings by differen-
tial interference phase contrast and phase contrast light microscopy showed that the

Figure 1. (a) This phase contrast light micrograph of an epoxy resin embedded skin scraping shows a
single giant cell. Note the numerous osmiophilic granules scattered throughout the cytoplasm
(×400).

(b) Serial section of (a). This transmission electron micrograph shows that the granules in (a)
are clumps of enveloped viruses mostly embedded in an electron-dense matrix. The nucleus (N) con-
tains only unenveloped viruses. An unaffected host cell at top left has complex cytoplasmic processes
that interdigitate with the giant cell (×6000).

(c) Detail from (b) above. Note the nuclear envelope. Desmosomes are shown (arrowed) at bottom
right. The cytoplasm of this giant cell contains the remnants of host cell organelles such as
mitochondria and rough endoplasmic reticulum. The majority of clumps of virus are bound by single
unit membranes (×12,000).

(d) These virus aggregates are details from the same giant cell and show the finely granular, elec-
tron dense matrix surrounding the virus particles. Some of the particles appear to be decayed or de-
graded. In some the capsids and cores are absent and the outer envelope appears broken open. Sheets
of membrane of unknown origin occupy the clump at lower left (×125,000).
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(a) 

(b) 

(c) 

(d)
giant cells seen by light microscopy and described by Richards & Buchanan (1978) were polynucleate (Fig. 1a). These giant cells were formed by the dissolution of the cytoplasmic membranes between cells with the consequent formation of syncytia. Subsequently, the nuclei within these syncytia fused to form a single giant nucleus which was usually oval with a maximum width of 60 μm and length of 100 μm. Numerous osmiophilic inclusion bodies of between 0.4 and 0.9 μm were scattered throughout the nucleoplasm (Fig. 1d). The nuclei were surrounded by a layer of cytoplasm 10–30 μm thick. The whole giant cell was therefore 70–130 μm in diameter and was approximately 1000 times the volume of the surrounding Malpighian cells of the epidermis. Serial section through individual giant cells enabled a picture of their ultrastructure to be built up. Details of the junction of giant cells with surrounding Malpighian cells are shown in Fig. 1b and c. The cytoplasm of the giant cells varied between vacuolato and highly granular in appearance and the cytoplasmic membrane showed multiple interdigitations with that of the overlying Malpighian cells. Desmosomes were frequently observed at points along the junction between giant cells and Malpighian cells (Fig. 1c). Nuclei similar to those of the surrounding Malpighian cells were occasionally found in the cytoplasm of these giant cells with their envelopes intact.

**Morphogenesis of the virus**

Electron microscopy of thin sections showed that the nuclei of giant cells contained finely granular material in which dense paracrystalline arrays of particles, 100 nm in diameter, were embedded. These resembled herpesvirus capsids with hexagonal or spherical outlines (Figs 1c, 2b & c, 3b & c). These dense clumps of virus corresponded with the nuclear inclusions visible by light microscopy and illustrated in Fig. 2a. Approximately half of these particles contained electron-dense cores and both the outer layer and core were found to correspond in size and shape to particles seen in negatively stained preparations from affected fish. In thin section the core appeared either as an electron-dense bar or as an electron-dense ring surrounding an electron-translucent centre. Capsids both with and without cores were arranged at the periphery of the nucleus adjacent to the nuclear envelope and enveloped particles were seen in large numbers immediately adjacent to the cytoplasm. Direct budding was not observed but virus particles in cytoplasmic vacuoles were invariably enveloped and it was probable that the envelope was acquired from the inner membrane of the nuclear envelope. Occasionally, the nuclear membrane appeared to rupture or to disintegrate so that unenveloped virus was seen in the cytoplasm; the capsids then appeared to acquire envelopes by budding into cytoplasmic vacuoles (Fig. 3b). The envelope surrounding the capsid in cisternae immediately adjacent to the nuclear envelope was often pear shaped or ‘tailed’. These perinuclear cisternae also contained ‘hour glass’ shaped envelopes without contained capsids but bearing the distinctive 18 nm long spikes associated with the outer surface of enveloped viruses (Fig. 3c). Enveloped virus particles in vacuoles further from the nucleus were more regular and these more bizarre forms were not seen.
Figure 2. (a) This phase contrast light micrograph is a 2 μm thick section of an epoxy resin embedded skin scraping from an affected turbot. The giant cell at centre is overlaid by a cap of Malpighian cells having microridges on their outer surface. Note the presence of numerous granular inclusion bodies in the nucleus (N) of the giant cell (×400). Section stained with 1% toluidene blue.

(b) Serial 50 nm thick section of the giant cell shown in (a). The granular areas in the nucleus in (a) are dense paracrystalline aggregates of unenveloped virus particles, 100 nm in diameter, some containing electron-dense cores. The cytoplasm of the cell contains cisternae packed with enveloped virus particles 200–220 nm in diameter (×9000).

(c) Further serial section of the giant cell shown in (a). The perinuclear cisternae appear to form continuous channels through the cytoplasm of the host cell (×12,000).
Figure 3. (a) For purposes of comparison with the virus particles shown in Fig. 4 this electron micro-
graph shows a herpes simplex virus particle and its envelope (× 200,000). Stained with 2% PTA, pH 7.

(b) Detail of the junction between nucleus and cytoplasm of the giant cell shown in Fig. 2. There is
some evidence that a process of budding of particles from the nucleus through the inner lamella of
the nuclear envelope gives rise to the enveloped viruses in the perinuclear cisternae. In places the
inner membrane of the nuclear envelope appears to have disintegrated and virus particles may acquire
their envelope by budding into cytoplasmic vacuoles (× 25,000).

(c) Further detail of the junction between nucleus and cytoplasm of the giant cell shown in Fig. 2.
Note the presence of numbers of pear shaped or tailed particles in the perinuclear cisternae. The
diameter of the enveloped viruses with their fringe of spikes is 200-220 nm. The mean diameter of the
capsids within the nucleus is 100 nm (× 125,000).
**Release of virus from giant cells**

Virus appeared to be released from the cell at the periphery of the cytoplasm either singly or in membrane-bound aggregates (Figs 1b & 2b). The cytoplasm of giant cells contained very large amounts of virus most of which appeared to lie within membrane-bound vacuoles. These were so numerous that it seems possible that they were cytoplasmic channels connecting the perinuclear cisternae to the exterior (Fig. 2b). The virus could be released by migration via these intracytoplasmic tubules from the nucleus to the exterior.

A proportion of giant cells contained membrane-bound aggregates of enveloped capsids embedded in a granular matrix (Fig. 1b & c). These clumps of virus were scattered throughout the cytoplasm and corresponded in size and shape to the osmiophilic granules seen by phase contrast light microscopy and illustrated in Fig. 1a. Frequently, these clumps included enveloped viruses that appeared to be undergoing degradation. The particles were often swollen with the outer envelope broken and the capsid either absent or poorly defined.

**The structure of the virus as seen in thin section**

Mature enveloped virions showed 5 concentric zones surrounding the core. The outer zone consisted of 18 nm long spikes which were attached to a trilaminar membrane forming the envelope or second zone (Fig. 3c). The envelope was separated from the capsid by a third, electron translucent layer containing various amounts of fibrillar material. The capsid (4th zone) was seen as an electron-dense ring that was frequently hexagonal in outline and this was separated from the core by an electron-translucent space (5th zone). The diameter of the enveloped particle, including spikes, was between 200 and 220 nm.

**Observations of negatively stained particles from affected fish**

Negatively stained preparations of skin scrapes with or without partial purification by centrifugation showed numbers of enveloped and unenveloped viruses closely resembling members of the herpesvirus group, but with some differences (Fig. 4a–e). The unenveloped capsids had a mean diameter of 100 nm (range 85–110 nm) with subunits of about 9 nm in diameter. Where stain had penetrated into the middle of a particle the subunits appeared as hollow tubes and this gave a castellated edge to the periphery of the capsid (Fig. 4a). Surface details were not seen clearly enough to establish the exact structure but the arrangement of subunits was very similar to that of the members of the herpes group. Despite careful searching and the use of standard staining conditions, details of the surface structure were never as clearly seen as they are on herpes simplex virus (Fig. 3a). Enveloped particles had a surface fringe of petal shaped spikes resembling those of coronaviruses. Their 18 nm length and the spacing between them were the same as the spikes seen in thin sections of giant cells. Frequently, the envelope was drawn out into a tail giving a tadpole
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appearance to the particle. An intact envelope appeared to be impervious to the stain and this prevented the surface details of the capsid being seen (Fig. 4a).

Discussion

Attempts are being made to culture *Herpesvirus scophthalmi* but, to date, no cell line has been found that will support the growth of this marine virus *in vitro*. Until this can be done full characterization of this new virus will not be possible. Nevertheless, the evidence we present indicates that the pathognomonic giant cells are virus 'factories', and that the virus produced has many of the characteristics of the herpesvirus group. This evidence consists of:

1. the formation of giant cells, syncytia and giant nuclei;
2. the development of intranuclear inclusions;
3. the assembly of capsids in the nucleus;
4. the envelopment of capsids at the inner nuclear membrane;
5. the architecture of the virus as shown by negative staining.

These points will be discussed in relation to known members of the herpesvirus group.

(1) The formation of giant cells

The primary cytopathic effect we have observed was that of virus-induced cell fusion. Polykaryocytosis is characteristic of herpesviruses (Pereira 1961; Roizman 1978). Ultrastructural evidence of cellular fusion came from observations of desmosomes at the junctions between giant cells and the surrounding Malpighian cells (Fig. 1c). Cook & Stevens (1970) found isolated desmosomes in the cytoplasm of cells infected with varicella zoster virus and interpreted this as evidence of virus-induced cell fusion. Further, apparently normal Malpighian cell nuclei were found within the cytoplasm of these giant cells but without the envelope of the giant cell nucleus. This suggested that nuclear recruitment was taking place and that giant cell nuclei were formed by the fusion of several nuclei following dissolution of the common membranes between the Malpighian cells. This is similar to the social behaviour of cells in cell

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**Figure 4.** (a) This group of virus particles includes one tailed particle having a fringe of 18 nm spikes. Stain has failed to penetrate this enveloped virus. The group of 4 unenveloped viruses show a definite substructure made up of hollow capsomeres. Compare these with the capsomeres on the herpes simplex virus in Fig. 3(a).

(b) Two viruses have dense cores that have not been penetrated by stain. The eccentric positioning of these cores may be an artefact resulting from centrifugation.

(c) Enveloped virus particle with a fringe of 18 nm spikes.

(d) These 3 broken particles show the hollow nature of the capsomeres that make up the capsid. The capsomeres are 9 nm in diameter.

(e) Tailed, enveloped virus. The envelope was frequently drawn out into a long tail during the preparative processes for negative staining.

All the above electron micrographs are at a magnification of 200,000.
cultures infected with herpesviruses described by Roizman (1978) and is compatible with observations made by Wolf & Darlington (1973) who carried out sequential studies of fish cell cultures inoculated with *Herpesvirus salmonis*.

(2) **The development of cytoplasmic and intranuclear inclusions**

Many of the features observed from phase contrast light microscopy of resin-embedded skin scrapings match those already described in a previous paper by Richards & Buchanan (1978) on the histopathology of this disease. For example, the osmophilic granules seen in the cytoplasm of some giant cells (Fig. 1a) corresponded in size and number to the Feulgen positive and occasionally PAS positive granules seen by light microscopy. Our ultrastructural observations showed that these granules consisted of membrane-bound clumps of enveloped viruses embedded in a finely granular electron-dense matrix. Similarly, the dense basophilic and Feulgen positive coarse granules described as one type of intranuclear inclusion could be correlated with dense paracrystalline arrays of unenveloped capsids enclosed within the nucleus of giant cells (Fig. 2a & c). The second type of intranuclear inclusion which consisted of eosinophilic, Feulgen negative bodies could be correlated with ultrastructural observations of areas in the nucleus devoid of virus particles and chromatin and containing only highly dispersed granular material. It seems probable that these inclusions were areas where former intense viral replication had exhausted the contents of a nucleus. Similar intranuclear inclusions in cell monolayers infected with herpes simplex virus have been described as scars left by earlier viral multiplication (Dulbecco & Eisen 1973). These intranuclear inclusions were considered by Pereira (1961) to be the most characteristic of the cellular lesions caused by viruses of the herpes group.

(3) **The replicative cycle of Herpesvirus scophthalmi**

Herpesvirus replication has been reviewed in detail by Roizman & Spear (1971). Our observations of the maturation sequence closely resemble those described from ultrastructural studies of other herpesviruses infecting animal cells (Smith 1963; Pinkerton, Sun, Henson & Neff 1964; Shipkey, Erlandson, Bailey, Babcock & Southam 1967; Nii, Morgan & Rose 1968; Stackpole 1969; Cook & Stevens 1970; Nazerian & Witter 1970; Nayak 1971; Wolf & Darlington 1971).

Incomplete capsids were often found in infected nuclei. Some appeared to be empty while others contained cores which were bar- or ring-shaped. Envelopment of the capsid was closely similar to that described by Wolf & Darlington (1971) for channel catfish virus. It occurred at the inner lamellae of the nuclear envelope and by budding into cytoplasmic vacuoles. Our observations support the concept proposed by Wolf and Darlington that envelopment can occur wherever the unenveloped capsid encounters host cell membrane. Further evidence in support of this maturation sequence came from observations of enveloped and partially enveloped virus particles, some having tail-like structures, in the perinuclear cisternae.
(4) The release of virus from giant cells

There seems to be general agreement that within the herpesvirus group envelopment takes place at the inner nuclear membrane but opinions differ regarding the release of virus from cells. Epstein (1962) showed that herpesvirus was released by budding at the cell membrane and Morgan, Rose, Holden & Jones (1959) and Nii, Morgan & Rose (1968) described the release of herpesvirus as a process of reverse phagocytosis. The mode of release through cytoplasmic channels was first described by Schwartz & Roizman (1969) and confirmed by Strandberg & Aurelian (1969) and Jasty & Chang (1972). Subsequent studies by Fong, Tenser, Hsuing & Gross (1973) showed that mature nuclear virus particles were first released into perinuclear cisternae and then travelled through cytoplasmic channels to the extracellular space. The results of the present study indicate that this method was the one by which *Herpesvirus scophthalmi* was released from giant cells. It seems likely that the cytoplasmic channels shown in Fig. 2b were modified areas of pre-existing endoplasmic reticulum (ER).

(5) Extracellular virus and a possible host cell reaction to the presence of the virus

Extracellular virus particles were frequently observed to be aggregated in membrane-bound clumps some of which contained numerous enveloped virus particles bound together in a finely granular matrix. Similar aggregations were observed in the cytoplasm of some giant cells and these bore a close resemblance to those described by McGavran & Smith (1965) who showed that clumping was due to virus-cell interaction. They noted that host cell lysosomes surround, engulf and obscure many cytomegaloviruses within the cytoplasm. Similar observations were made by Reubner, Hirano, Slusser, Osborn & Medearis (1966) of cytomegaloviruses embedded in electron-dense material having many of the characteristics of lysosomes suggesting that a host-cell reaction against virus produced in its own nucleus may have taken place. They considered that this reaction could be pathogenetically important in long term infections. The present study has shown that membrane-bound aggregates of virus in giant cells show the virus in differing stages of dismemberment and decay as though under attack from lysosomal enzymes. Further cytochemical studies are required to determine the nature of the matrix that surrounds the viruses.

(6) The morphology of the virus as shown by negative staining

Negative staining has shown that the appearance of the virus accords closely with that of other members of the herpesvirus group as illustrated in comparative studies by Madeley (1972) and Roizman & Furlong (1974). However, the outer layer of spikes on the envelope appears to be unique to *Herpesvirus scophthalmi* in that they are approximately twice as long as those reported by Wildy, Russell & Horne (1960); McCracken & Clarke (1971) and Fong *et al.* (1973). The most clearly defined spikes were described by Wildy *et al.* as being 8–10 nm long and 5 nm intervals apart.
The other architectural components of the virus were typical of the herpesvirus group as a whole. In a review, Roizman & Spear (1971) noted that there is general agreement that the typical herpes virion consists of a core 25-30 nm in diameter, a capsid consisting of 162 capsomeres and an envelope (with or without spikes) and thus can be defined as an enveloped nucleocapsid. The present study has shown that the turbot virus possessed a core having a diameter of 25-30 nm and that was compatible with a toroid when seen in thin section. The core was bound by a capsid that was frequently hexagonal in outline and having a mean diameter of 100 nm and a definite substructure of hollow capsomeric units. These were less well defined than in most herpesviruses but they were comparable in size and number to the characteristic hollow herpesvirus capsomeres.

The total diameter of mature enveloped particles was 200-220 nm as compared with 160-180 nm for the herpesvirus group as a whole but this was due to the fringe of spikes surrounding the envelope.

The relative ease of diagnosis and the unfortunate abundance of infected fish suggest that this new virus would provide an ideal subject for comparative studies within the herpesvirus group.

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