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Virus-like particles associated with cytopathology in the digestive gland epithelium of scallops *Pecten novaezelandiae* and toheroa *Paphies ventricosum*

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ABSTRACT: Apparent replication of small DNA-negative virus-like particles (VLPs) is described from digestive and secretory (= basiphil) cells of scallops *Pecten novaezelandiae*, Reeve, 1853 and toheroa *Paphies ventricosum* (Gray, 1843) sampled during mass mortalities, and compared with apparently healthy individuals. In scallop digestive cells with putative VLPs, endocytotic and smooth membrane vesicles increased, endoplasmic reticulum (ER) proliferated, and VLPs 22 to 30 nm across were seen in an orderly array on the surfaces of the outer nuclear membrane and along ER. Proliferating ER membranes, lined with VLPs and enclosing a dense matrix, were arranged in a reticulated configuration. The ER cisternae dilated to form vacuolar inclusions (VI) containing elongated bodies, spherical in section, in a flocculent matrix which were ornated with VLPs arrays on the external membrane. Enclosed bodies also formed by budding of cytoplasm into the VI. In scallop secretory cells VLPs replaced ribosomes on ER, and ER cisternae dilated, but VI seldom formed. Toheroa diverticular epithelium showed similar changes, but secretory cells differed in that the outer membrane of the nucleus and Golgi cisternae, rather than ER, proliferated. In addition, complete VI were apparently not formed. The cytological changes observed in both bivalves are similar to those associated with enteroviruses (Picornaviridae) and caliciviruses. The possible role of VLPs in bivalve pathology is discussed.

KEY WORDS: Scallops · clams · Diverticular disease · Virus-like particles · Picornaviruses · Caliciviruses

INTRODUCTION

The New Zealand scallop *Pecten novaezelandiae* dredge fishery produces 3000 to 4000 t wet weight of scallops per annum (Bull 1991), but intermittent mortalities of up to 39% per annum have been reported among wild stocks (Bull 1976). Sporadic population crashes have made the fishery very difficult to manage, and have resulted in requests to re-stock areas with scallops from other regions. Attempts at growing scallops under culture conditions have also resulted in mass mortalities. Similarly, toheroa *Paphies ventricosum*, a large clam collected recreationally, also experience population crashes. Subsequent examination of moribund scallops and toheroa has only revealed diverticular lesions, similar to those reported as part of the normal cycle of degeneration and renewal of diverticular epithelium reported by Henry et al. (1991) from *Pecten maximus*, and associated with algal blooms in *Pecten alba* in Australia (Parry et al. 1989).

The diverticular lesions in scallops and toheroa resemble those lesions in the moribund mussels *Perna canaliculus* and *Mytilus galloprovincialis* that have been associated with the presence of small RNA viruses in diverticular epithelial cells (Jones et al. 1996). In view of the similarities in scallop and toheroa epizootics and diverticular pathology to those associated with RNA viruses in mussels, scallops and toheroa from mortalities and from apparently healthy individuals were examined by transmission electron microscopy for virus-like particles similar to those reported from mussels. The possible identity and role of these small RNA viruses is considered.

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MATERIALS AND METHODS

Between December 1990 and April 1993, 264 dredged wild scallops and 12 moribund scallops held under culture conditions, 23 to 110 mm shell height, were examined for disease. Whole body sections through the mantle, gills, gonad, and digestive organ were fixed in Davidson's fixative and stained routinely with haematoxylin and eosin (H&E). Sections were also stained with Feulgen-picro-methyl-blue for DNA (Farley 1969), Oil Red O for lipids (Lynch et al. 1969), Perl's stain for iron, Periodic Acid-Schiff (PAS) for glycosaminoglycans, Schmorl's stain for lipofuchsin, and were bleached for melanin using 0.25% aqueous potassium permanganate (Luna 1968).

For transmission electron microscopy (TEM), excised digestive tissues were fixed in 2.5% glutaraldehyde in 0.22 µm filtered seawater (FSW) for 1 h, washed twice in FSW, post-fixed in 1% OsO_4 for 1 h, dehydrated in ascending (50 to 100%) ethyl alcohol, embedded in Araldite, thick-sectioned and stained with 1% toluidine blue in 1% borax solution, or ultrathin-sectioned and stained with 5% uranyl acetate for 10 min and 5% lead citrate for 5 to 6 min, and examined on a Philips 420ST TEM.

Toheroa *Paphies ventricosum* from mortalities among wild stocks in April 1991 (n = 6), January 1993 (n = 6) and November 1994 (n = 4), and healthy toheroa collected in April 1991 (n = 12) were fixed and prepared for TEM as described above.

Cell organelle terminology follows Henry et al. (1991).

RESULTS

Scallops *Pecten novaezelandiae*

No lesions were apparent macroscopically, but under the light microscope all scallops had lesions in the epithelial cells of the distal sections of digestive diverticulae. In H&E sections the shape of most diverticulae appeared normal, but much of the diverticular epithelium resembled empty compartments containing yellowish irregular material (Fig. 1) In the most affected scallops the entire tubule was composed of these cell 'ghosts' (Fig. 1), or the compartments had broken down exposing the underlying basement membrane (Fig. 2). Damage was extensive and severe in many of the scallops examined and larger scallops tended to possess a higher proportion of affected cells. Feulgen-picro-methyl blue failed to reveal inclusions that might suggest a DNA-positive aetiological agent. The yellowish amorphous material was negative with other stains, except for moderate amounts of lipid and PAS-positive substance.

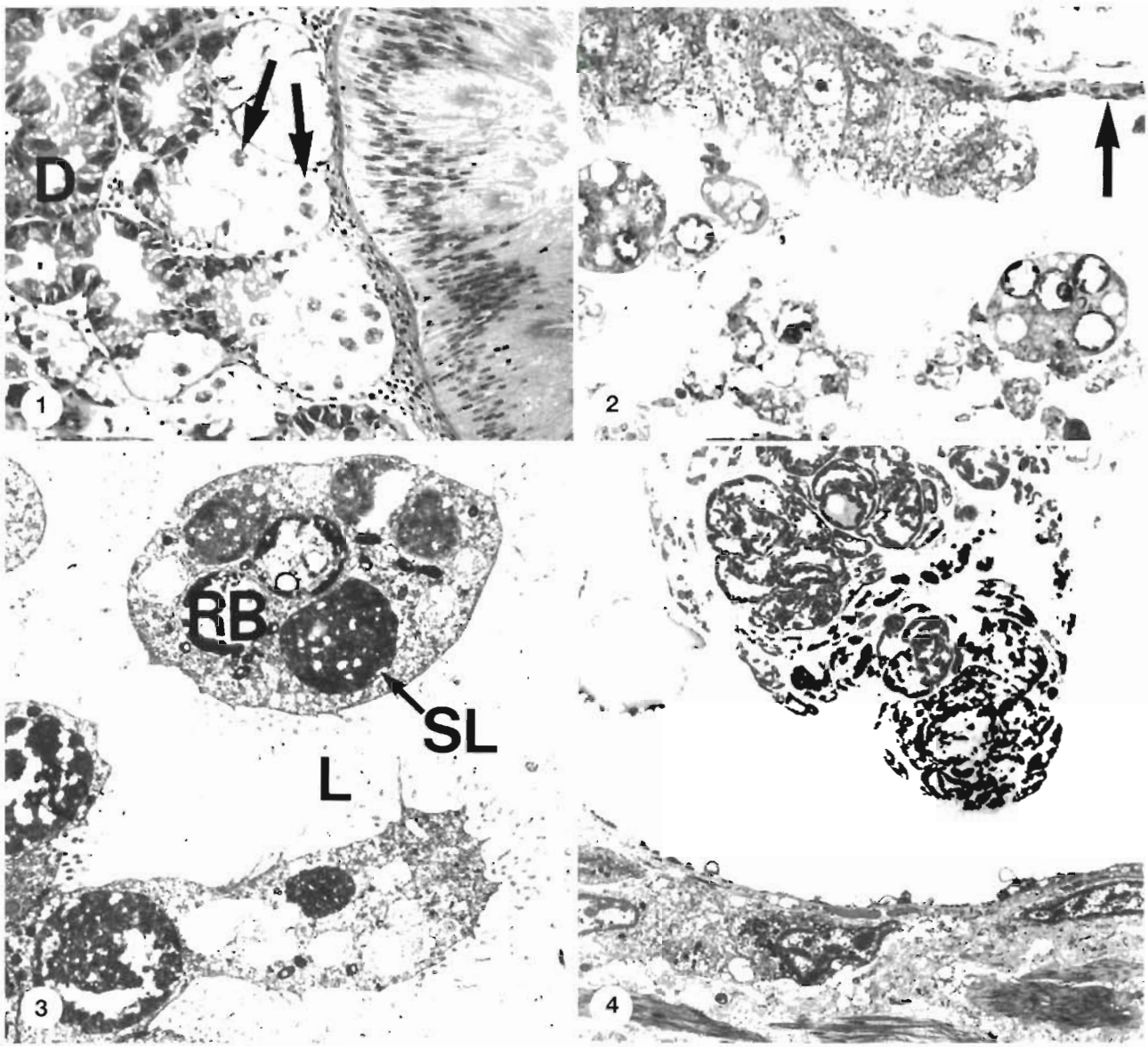
At the TEM level, diverticular epithelium of healthy scallops comprised digestive cells and secretory (= basiphil) cells. Digestive cells (DC), which were most abundant on the luminal surface, had an irregular nucleus with a prominent nucleolus and sparse marginated heterochromatin. Coated endocytotic pits between the microvilli of the brush border appeared to form smooth-membraned endocytotic vesicles (EV) in the apical cytoplasm. Several Golgi profiles with electron-dense content in the cisternae, short strands of smooth endoplasmic reticulum (ER), a few vacuoles and lipid droplets, residual bodies, secondary lysosomes and elongated mitochondria with a dense matrix were also present.

Secretory cells (SC) were more common near the basal lamina, and had a nucleus similar to DC. However, the cytoplasm possessed long meandering strands of rough endoplasmic reticulum (rER), a few dense perinuclear Golgi arrays, membrane-bound spherical secretory granules with a finely granular moderately dense homogenous content, and large numbers of ribosomes giving the SC a dense appearance.

In light, possibly early, lesions, the brush border of DC was ragged, DC protruded into the diverticular lumen and detached (Fig. 3) and disintegrated, leaving necrotic cellular debris and clusters of residual bodies in the lumen (Fig. 4). Sometimes the DC disintegrated *in situ* in the epithelium to leave a compartment with clustered residual bodies. Detachment exposed the underlying basement membrane (Fig. 4) and, as the tubule became progressively affected, remnant secretory cells became isolated and detached, until the whole tubule lacked an epithelium.

Also in apparently early lesions, DC Golgi became less apparent, the number and size of EV increased, a few more smooth ER profiles were present, and the brush border became fragmented and disorganized. In possibly later lesions, ER and arrayed virus-like particles (VLPs) proliferated. Many smooth-membraned vesicles (SMVs) (Fig. 5), some with duplicated or multiple membranes (Fig. 6), accumulated in the cytoplasm. VLPs, usually 22 to 30 nm but <36 nm in diameter, were arrayed along the cytoplasmic surface of the nuclear membrane and along ER (Fig 7), often in proximity to mitochondria (Fig. 8). VLPs were also observed free in the cytoplasm, sometimes in cytoplasmic projections from the surface (Figs. 9 & 10).

Dense flocculent material occurred in the cisternae of ER lined with VLPs, and ER membranes appeared to rearrange to form a reticulated structure containing a dense matrix (Fig. 11). Dilated matrix-filled cisternae appeared as vacuoles with a floccular content and with VLPs arrayed on the cytoplasmic surface (Fig. 12). The surrounding cytoplasm appeared to bud into the vacuole to form spheres and elongated bodies of cytoplasm with



Figs. 1 to 4. Histological changes. Fig. 1. H&E section showing normal epithelium of the main gut, a normal digestive diverticulum (D) and affected diverticulae with empty compartments, some containing yellow material (arrows) (×210). Fig. 2. Section through partly affected tubule showing apparently normal digestive cells (DC) with a brush border, rounded DC and necrotic debris in the lumen, and bare basement membrane (arrow) where the epithelium has been lost (×1050). Fig. 3. Protrusion into the lumen (L) and sloughing by DC with prominent residual bodies (RB), vacuoles and secondary lysosomes (SL) (×6060). Fig. 4. Surface of a diverticulum devoid of epithelium, leaving a bare surface overlying the basal lamina, and clumped RB in the lumen (×3660)

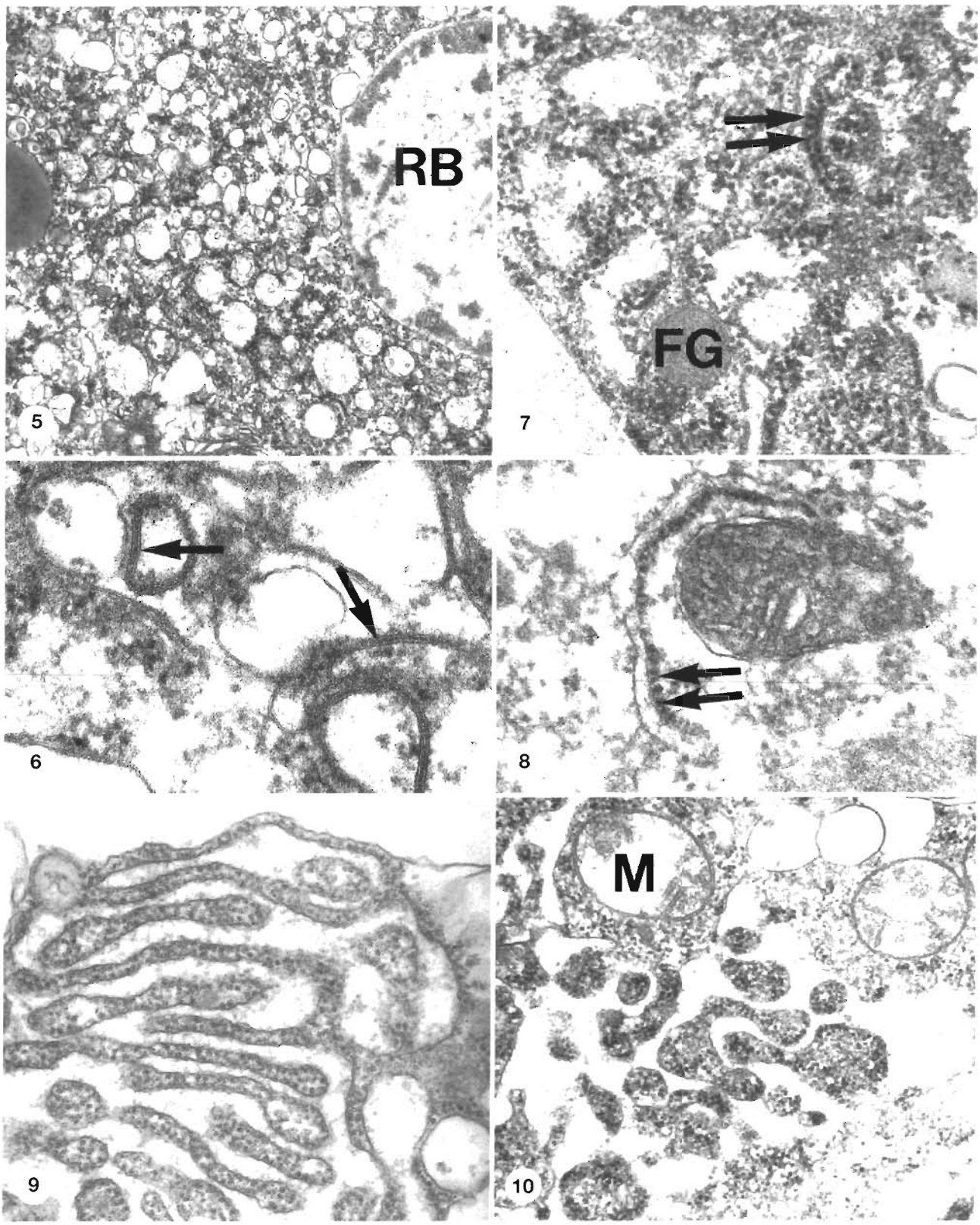
VLPs arrayed along the inside surface to form vacuolar inclusions (VI) (Figs. 12 & 13). Mitochondria were frequently swollen, with detached cristae (Fig. 10). Membrane-bound ovoid or pleomorphic bodies with a fine granular content were present in the cytoplasm (Fig. 7).

Sloughed DC were rounded and possessed few fragmented microvilli. Their cytoplasm was densely packed with VIs, free VLPs, reticulated structures, secondary lysosomes, SMVs, lipid droplets, EVs, and clustered residual bodies (Fig. 14).

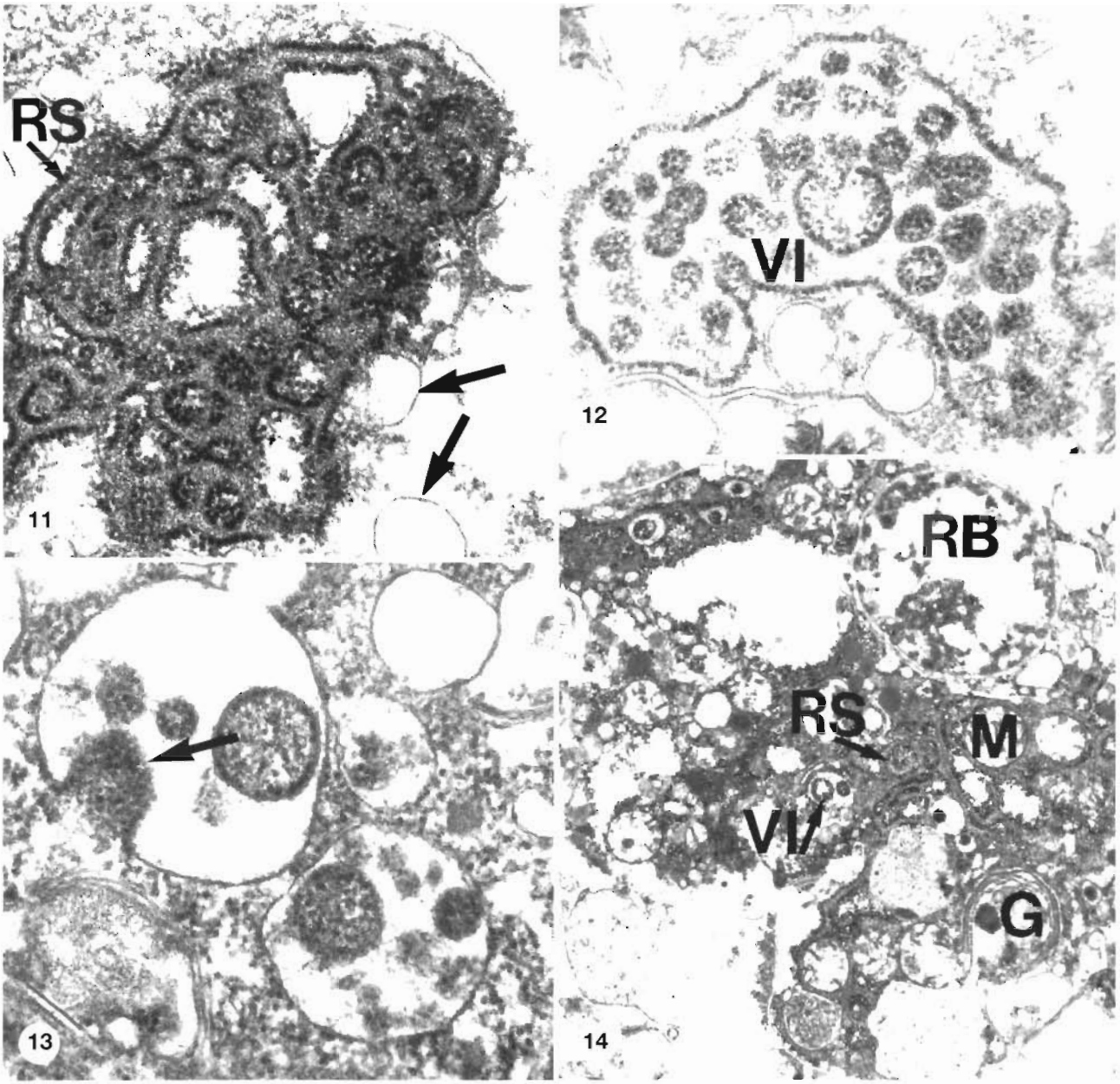
In SC the only changes observed were the replacement of 15 nm ribosomes on rER with VLPs, and dilation of ER cisternae containing flocculent material.

Toheroa *Paphies ventricosum*

The cytology of normal SC was similar to that of scallops. DCs differed from scallops in the greater density of EVs, and the denser more granular appearance of secondary lysosomes.



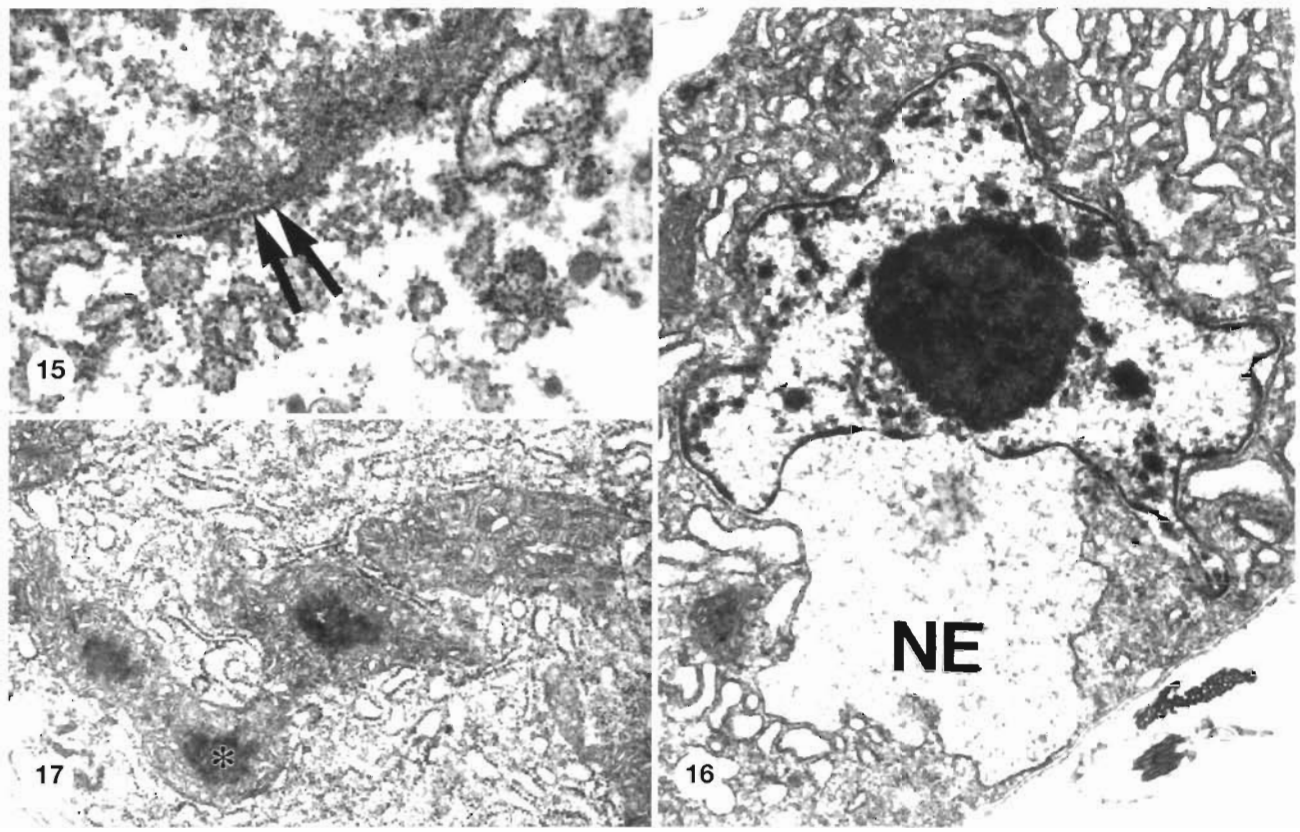
Figs. 5 to 10. Cytoplasmic changes in digestive cells. Fig. 5. Cytoplasm of a sloughed digestive cell (DC) with many cytoplasmic vacuoles, a residual body (RB) and lipid droplet (×21 970). Fig. 6. Detail of cytoplasmic vacuoles showing duplication of the bounding membrane (arrows) (×105 000). Fig. 7. Endoplasmic reticulum (ER) cisternae with arrayed virus-like particles (VLPs) (arrows) and a body containing a fine granular matrix (FG) within the cytoplasm (×64 800). Fig. 8. ER with VLP on the surface nearest a mitochondrion (×82 000). Fig. 9. Finger-like projections from the cell surface enclosing VLPs (×53 080). Fig. 10. Surface of adjoining DCs showing cytoplasmic projections containing VLPs, and swollen mitochondria (M) (×44 080)



Figs. 11 to 14. Reticulated structures and vacuolated inclusions in digestive cells (DC) Fig. 11. Reticulated structure (RS) with dense matrix in the cisternae, and smooth-membranes vesicles (SMVs) (arrows) (×54 760). Fig. 12. Vacuolar inclusion (VI), showing spherical bodies within the vacuole containing flocculent material and virus-like particles (VLPs) (×54 680) Fig. 13 Cytoplasm with VLPs (arrow) apparently budding into a VI (×64 720). Fig 14. Necrotic digestive cell with a residual body (RB) surrounded by a dense RS, Golgi (G), vacuolar inclusions (VI) and swollen mitochondria (M) (×17 500)

In apparently infected DC, VLPs 22 to 36 nm across were arrayed along the outer nuclear membrane and dilated ER cisternae containing flocculent material, particularly in the perinuclear region (Fig. 15) However, VI were not observed. SMVs were less common in intact cells than in scallops, but after lysis large numbers of ovoid membranes remained bearing VLPs 20 to 24 nm across, similar to those observed in scallops.

In SC containing VLPs, the outer nuclear membrane was sometimes detached and enlarged to form a nuclear extrusion (Fig 16). Large SMVs were frequently observed near Golgi cisternae, and may have derived from them. A fine granular matrix occurred in the centre of mitochondria in some SC with cytoplasmic VLPs (Fig 17). Cisternae of rER denuded of ribosomes and with VLPs arrayed along them were often dilated and contained flocculent material (Fig 17) The



Figs. 15 to 17 Toheroa infections Fig 15 Edge of nucleus with virus-like particles (VLPs) along the nuclear membrane (arrows) and around dilated endoplasmic reticulum (ER) cisternae containing flocculent material (×39 370). Fig. 16. Nucleus of secretory cells (SC) with proliferation of the outer nuclear membrane to form a nuclear extrusion (NE) (×14 820). Fig. 17. Mitochondria in SC showing the central fine granular matrix (*) (×21 580)

early stages of VI formation were occasionally observed.

In both bivalve hosts, extracellular VLPs were not observed and some sloughed DC showed none of the signs of putative viral infection reported above.

DISCUSSION

Studies on viral pathogens of molluscs are limited by the lack of molluscan cell-lines in which to culture the putative viruses, necessitating other approaches, such as the use of TEM. TEM has its limitations as replication may not involve much visual cytopathology. In addition the process of replication may not only differ between and within groups, but also *in vivo* and *in vitro* or in relation to virulence. Finally, small viruses are structurally at the limits of resolution. The latter is true of this study, and the putative VLP are the same size as β-glycogen (30 nm), only slightly larger than ribosomes (15 nm) and may array along ER in a manner very similar to ribosomes along rER. Despite this, the VLPs in this study can be distinguished from β-glycogen by their affinity for membranes, and ribosomes on the basis of size (22 to 36 nm). More importantly, many of the structures reported here resemble those involved in replication of RNA viruses.

The putative virogenic inclusions (VI, reticulated structures) reported here are unlike the organelles of eukaryotic cells, and the VLP arrayed along ER could also be distinguished from rER by the greater electron density of VLP. Similar non-ribosomal arrays occurred along the nuclear membrane, unlike ribosomes in eukaryotic cells. The large number of cytoplasmic vesicles and vacuoles in sloughing and sloughed digestive cells were not observed in healthy cells. None of these structures, including VI, have been reported from normal epithelium of bivalve diverticulae (Owen 1970, Pal 1971, 1972, Henry 1984a, b, Henry et al. 1991) or other molluscs (Nelson & Morton 1979).

The Picornaviridae comprises the enteric viruses (polioviruses, enteroviruses, echoviruses, coxsackieviruses), rhinoviruses, which are a cause of the common cold, *Cardiovirus*, which causes encephalomyocarditis, and aphthoviruses which cause foot-and-mouth disease and similar diseases (Melnick 1983). At

22 to 30 nm they, and caliciviruses, are similar in size to the VLPs seen here, and some picornaviruses and caliciviruses show similarities in replication to bivalve VLPs.

In cells infected with mengovirus (Amako & Dales 1967) or poliovirus (Bienz et al. 1980, 1983, Hashimoto et al. 1984) large numbers of smooth membrane vesicles develop in the central region of the infected cell, resembling the SMVs reported here. Polioviral protein (Bienz et al. 1980, 1983) or VLPs (Hashimoto et al. 1984) accumulate around these vesicles, which are part of a poliovirus replication complex (Bienz et al. 1987, 1992) in which viral RNA is synthesized (Bienz et al. 1980, 1987). In poliovirus infected cells, a smooth membranous tubular network develops that encloses fingers of cytoplasm in which viruses subsequently develop, resembling VI in this study (Dales et al. 1965). In poliovirus (Mattern & Daniel 1965) and coxsackievirus B5 (Shahrabadi & Morgante 1977) infected cells, similar configurations result from proliferation of the outer lamina of the nuclear membrane to form multilayer membranous structures containing finger-like projections of cytoplasm. The subsequent accumulation of coxsackie B5 viruses along the outer membrane and development of viruses within the membrane-bound vesicles results in configurations indistinguishable from scallop VI (Shahrabadi & Morgante 1977; Figs. 2a & 3 therein). Duplication of SMV membranes, similar to those in Fig 6, also occurs in mengovirus infection (Amako & Dales 1967). Swelling of mitochondria has been reported in calicivirus (Studdert & O'Shea 1975), poliovirus (Hashimoto et al. 1984), and coxsackievirus (Rabin et al. 1964) infections, but, like the increase in EV, is likely to be related to cell death rather than the presence of an infectious agent. The SMVs may have developed from modified EVs, but this was not observed.

Enterovirus virions may be arrayed along cisternae to give the superficial appearance of rER (Yilma & Breese 1980), and several studies show that enteroviruses may be released into spherical to ovoid or elongated structures, as seen in VI (Dales et al. 1965, Wroblewska et al. 1977, Yilma & Breese 1980, Tucker et al. 1993). In particular, the surface cytoplasmic projections containing VLPs (Figs. 9 & 10) resemble those caused by aphthoviruses (Yilma et al. 1978, Polatnick & Wool 1983), which may be formed by budding into vacuoles (Wool et al. 1982) in a manner identical to budding into VIs. During calicivirus infections, Golgi cisternae may form fine granular dense bodies (Love & Sabine 1975) similar to those observed here (Fig. 7) and in coxsackievirus infections (Jézéquel & Steiner 1966). In coxsackievirus infections, Golgi cisternae proliferate to form membranous vesicles (Jézéquel & Steiner 1966) resembling those in toheroa SC. The proliferation of the outer nuclear membrane to form a nuclear extrusion (Fig. 16) is similar to nuclear membrane extrusions in calicivirus (Love & Sabine 1975), and enterovirus (Rabin et al. 1964, Mattern & Daniel 1965, Shahrabadi & Morgante 1977) infected cells. The VLPs may be mature virions that are shed in the spherical to elongated bodies in VI, or in surface cytoplasmic protrusions, as in some enteroviruses (Dales et al. 1965, Wroblewska et al. 1977, Yilma & Breese 1980, Tucker et al. 1993) and aphthoviruses (Yilma et al. 1978, Wool et al. 1982, Polatnick & Wool 1983).

Picornaviruses differ from bivalve VLPs in usually forming crystalline aggregates or matrices in infected cells (Dales et al. 1965, Yilma & Breese 1980, Rodriguez et al. 1983). The lack of crystalline arrays in the bivalves studied here may indicate lack of virion maturation, or be related to the cell type infected as poliovirus forms crystalline arrays *in vitro*, but not *in vivo* (Hashimoto et al. 1984). Coxsackievirus infections may also differ in the involvement of the host cell nucleus in replication in some species (Rabin et al. 1964, Jézéquel & Steiner 1966). Caliciviruses may differ in their association with fibrils (Love & Sabine 1975, Studdert & O'Shea 1975), which was not observed here.

Despite these differences, a viral aetiology is suggested because the VI, RS, ovoid membrane-bound bodies with fine granular matrix and reduplicated SMV membranes are not known from the digestive epithelium of scallops (Henry et al. (1991), but do occur during replication of several picornaviruses and some caliciviruses.

It would be premature to conclude that cellular alterations associated with the VLPs are the cause of scallop or toheroa mortalities, despite the common observation of putative virogenic structures among many sloughing and necrotic cells. In affected bivalves, the sloughing of DC lacking VLPs may result from loss of structural integrity following loss of infected cells. However, diverticular epithelial cell renewal appears to be a normal process in molluscs (Nelson & Morton 1979, Henry et al. 1991). VLPs may be irrelevant to degeneration, or they may alter the kinetics of degeneration and renewal, leading to disease. Further studies are needed to identify the VLPs, to fulfil Koch's postulates and show the VLPs cause diverticular disease, and show such disease is the cause of the large scale mortalities.

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