Virus-associated Submandibular Gland Duct Cell Cytomegaly in a European Hedgehog, *Erinaceus europaeus* L.

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Cytomegaloviruses undergo the classical morphologic stages of replication in hedgehog submandibular gland duct cells. A profound cytomegaly is associated with the infection. These features indicate that the hedgehog may be an excellent model in which to study the biology of cytomegalovirus-salivary gland interactions.

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Introduction.

A unique form of cellular hypertrophy, believed to be a consequence of infection, was first described in 1904 by Jesionek and Kiolemenoglou[1] and by Ribbert.[2] From that time on, this particular form of cellular hypertrophy, ultimately termed "cytomegalia,"[3] was frequently described as occurring in salivary glands (see reviews[4,5]). The nature of the infection associated with pronounced cellular enlargement and characteristic nuclear inclusions remained controversial until evidence was presented in 1926 that a filterable virus was the causative agent.[6] An interval of three decades passed before the infective virus, isolated from mouse salivary glands, was successfully propagated in tissue culture,[7] with its designation as a "cytomegalovirus" following six yr later.[8]

The interim designation of cytomegaloviruses as salivary gland viruses[9] is a strong affirmation of the association of this particular viral group with salivary glands. As such, fine structural studies of naturally occurring or induced cytomegalovirus salivary gland infections in a variety of species have appeared in the literature (mouse,[10-13] shrews,[14] guinea pig,[15] marmoset,[16] and squirrel monkey[17]). This report presents our morphologic findings concerning what appears to be a cytomegalovirus infection with attendant profound cytomegaly in the submandibular salivary gland of a European hedgehog.

Materials and methods.

The submandibular glands of nine European hedgehogs – six females and three males (*Erinaceus europaeus* L.) – were examined in a survey of acinar and ductal cell ultrastructure and histochemistry.[18,19] The animals were killed by an overdose of pentobarbital. The submandibular glands were removed, cut into small blocks, and subsequently fixed in 1.25% glutaraldehyde and 2% paraformaldehyde in phosphate buffer (pH 7.3) for three h at room temperature. Tissues were post-fixed in phosphate-buffered 1% osmium tetroxide, were dehydrated through ascending concentrations of ethanol followed by propylene oxide, and were embedded in Epon 812. Semi-thin (1.0 μm) sections were stained with alkaline (pH 9) 1% toluidine blue. Thin (50-60 nm) sections were collected on carbon-reinforced, Formvar-coated grids, stained with uranyl acetate and lead citrate, prior to ultrastructural examination.

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Results.

The gross anatomical features of the submandibular glands from all the animals studied appeared similar. Foci of hyperemia, necrosis, or fibrosis were not observed during the slicing of the glands for fixation. In one animal of the nine studied, semi-thin (1.0 μm) sections of the gland contained occasional foci of cellular gigantism with individual cells that formed the foci being several times larger than either normal duct or acinar cells (Fig. 1). These cellular formations were circumscribed by loose connective tissue which contained a mononuclear cell infiltrate. The only other distinctive alteration in the gland structure was that granular striated ducts[19] adjacent to the clusters of hypertrophied cells lacked secretory granules (Fig. 1).

A fortuitous section revealed that the hypertrophied cells comprised a portion of the epithelial wall in a granular striated duct (Fig. 2). Adjacent duct cells, although lacking secretory granules, were of normal size and possessed the distinctive ultrastructural features – *i.e.*, complex interlocking basal folds; equatorial nuclear position; a small, supranuclear Golgi complex; and small cisterns of granular endoplasmic reticulum–that are characteristic of this cell type.[19] Examination of the altered ducts demonstrated greatly enlarged cells that had a faintly striated cytoplasm in which small, deeply-stained cytoplasmic granules were widely distributed. When duct lumens were present in the section, the cytoplasmic granules were observed to occupy the luminal half of the cytoplasm (Fig. 3). Each cell had an enlarged nucleus that contained a distinctive reticulate or

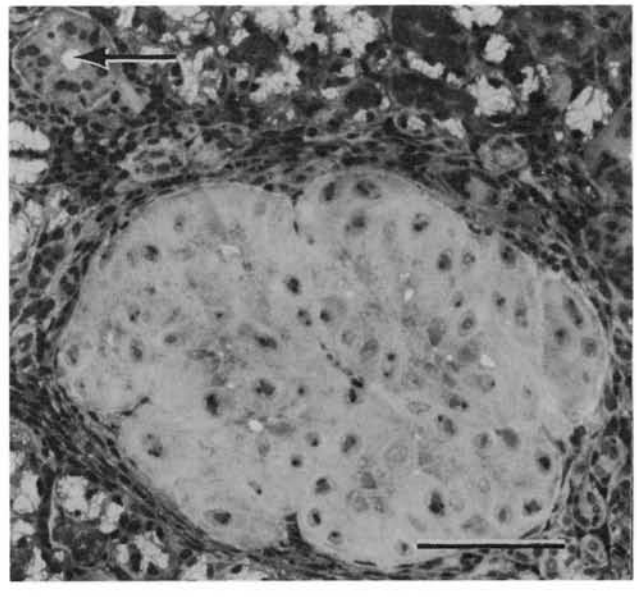

Fig. 1 – Photomicrograph of a region exhibiting cytomegaly within the submandibular gland. The cells are surrounded by a thin rim of cellular connective tissue. A normal-sized striated duct is indicated by the arrow (X 156). Bar = 100 μm.

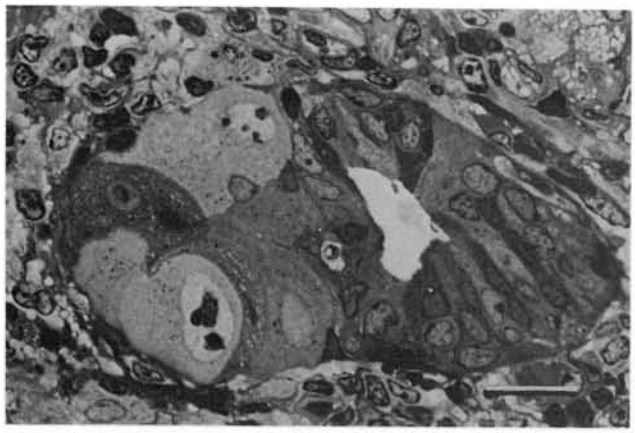

Fig. 2 – A partially tangential section of a duct illustrating normal and hypertrophied cells comprising the epithelial lining. The surrounding connective tissue is infiltrated by mononuclear cells (X 625). Bar = 20 μm.

honeycomb-shaped inclusion surrounded by angular, deeply-stained satellite inclusions.

The ultrastructure of such nuclei is presented in Figs. 4 and 5. The reticulate inclusion which dominated the nucleoplasm was composed of condensed granular, interconnecting cords. Its interstices were filled with both a dispersed, punctate material and forming or complete viral nucleocapsids. Large numbers of closely packed circularly or hexagonally shaped nucleocapsids were located around the periphery of the inclusion. While a small percentage of the capsids were either empty or filled with an electron-dense core, most capsids were filled with a circular nucleoid. The satellites of the inclusion were composed of an electron-dense fibrillar or granular material. These satellite inclusions did not have any distinctive viral structures associated with them. Rarely were nucleocapsids observed "budding" through the inner nuclear membrane (Fig. 6). We were unable to determine whether such "budding" nucleocapsids acquired an envelope as a consequence of their entry into the cytoplasm through the nuclear membrane.

The acquisition of the viral envelope took place principally in the paranuclear Golgi zone. In this region, nucleocapsids appeared progressively to indent tubular or circular vesicles, and, as a result, began to acquire an outer membrane and an interposed condensed matrix (Fig. 7). As the nucleocapsid further indented the vesicle, matrix accretion progressed circumferentially, as did the encasing of the virus by the vesicle membrane (Fig. 8). The nucleocapsid was usually located eccentrically within the envelope matrix. Numerous apparent defects in the envelopes, characterized by electron-lucent zones in the matrix and deformed outer membranes, were observed (Fig. 9).

The apical one-third to one-half of the cytoplasm in infected cells was populated by vesicles filled with enveloped virions (Fig. 10). Some of these vesicles also contained a membrane-enclosed electron-dense matrix, in addition to the viral particles. These virus-containing vesicles were the ultrastructural equivalent of the abundant small cytoplasmic granules observed with the light microscope. Viral discharge into the gland lumen was not encountered, although solitary viral particles were observed within duct lumens (Fig. 11).

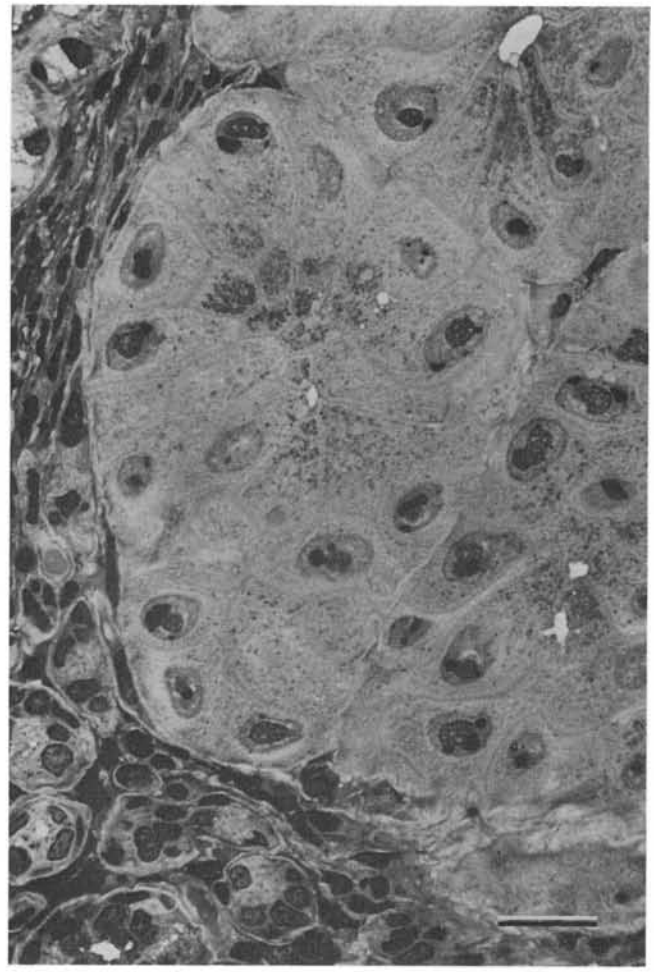

Fig. 3 – Photomicrograph illustrating the features of cytomegaly in the submandibular gland. The cytoplasm of the enlarged cells is stippled due to the presence of cytoplasmic granules. The nuclei contain a large reticulate inclusion surrounded by darkly staining, angular satellite inclusions (X 625). Bar = 20 μm.

Discussion.

The observed cytomegaly, the distinctive intranuclear inclusion ("honeycomb,"[3] "skein-like,"[20] "reticular,"[21] and nuclear inclusion "subunits"[22]), and the ultrastructure of the virus in the afflicted cells all warrant the diagnosis of a cytomegalovirus (CMV) infection. In the hedgehog, viral replication and cytopathic change occurred within the ducts. This location is similar to CMV infections in the guinea pig[23] and monkey.[24] Acinar infections are reported to be more common in the mouse,[11] hamster,[5] and chimpanzee.[25] In the rat, both ducts and acini may be affected,[26] while in the mole, "terminal tubules" have been reported to be the site of infection.[27] "Terminal tubules" can be inferred to be secretory acini, based on the authors' descriptions.[27] A subsequent study in shrews and moles has demonstrated viral replication in both acini and ducts.[14] We also observed that the distinctive duct cell secretory granules[19] are lost from adjacent duct cells as a consequence of infection. The reason for this cytoplasmic change in contiguous, but apparently uninfected, cells is not known.

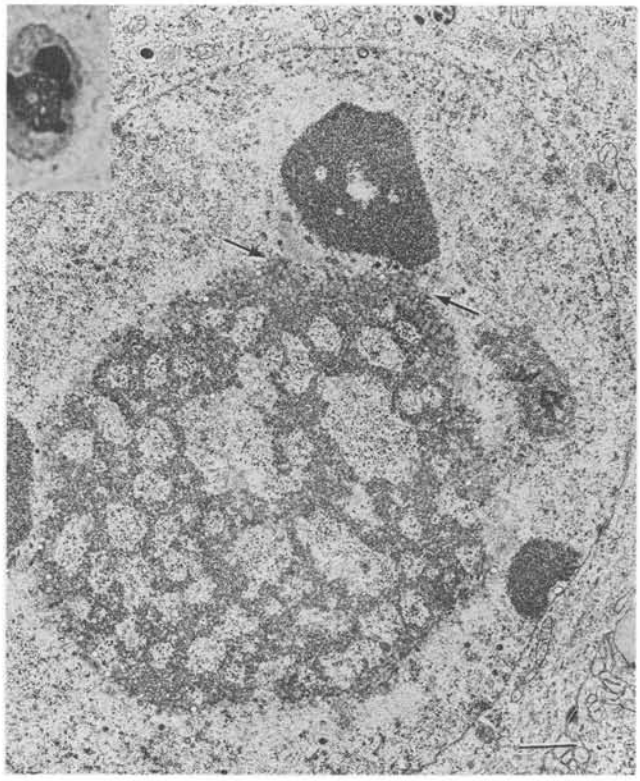

Fig. 4 – The cytology of the intranuclear inclusions in an infected cell is illustrated. The central reticulate inclusion is surrounded by clusters of nucleocapsids, some of which are indicated by arrows. The electron-dense satellite inclusions do not appear to be associated in any unique way with the nucleocapsids. The inset illustrates the light microscopic appearance of the nucleus shown in this electron micrograph (X 13,700; inset; X 2000). Bar = 1 μm.

The sequence of viral replication observed in the hedgehog duct cells is similar to that reported by others both *in vivo* and *in vitro*. Nucleocapsids appeared to be preferentially associated with the nuclear reticulate inclusion.[20,28] "Budding" through the nuclear membrane[15,28] was also observed. In agreement with the findings of Kanich and Craighead,[28] we noted electron-dense material opposite

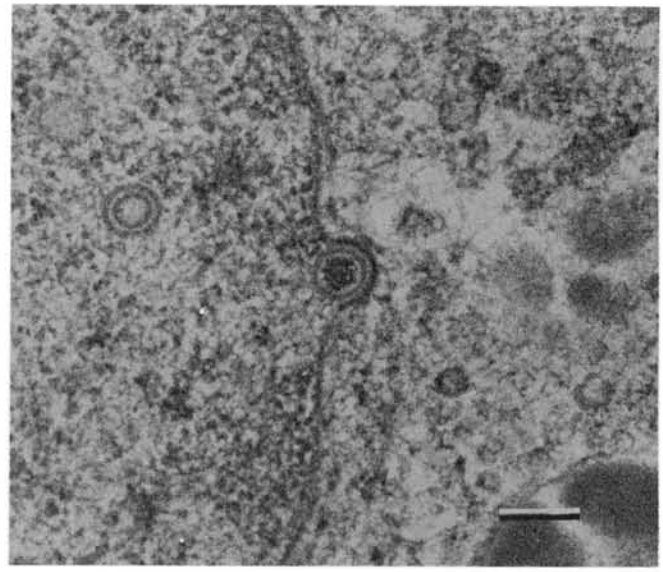

Fig. 6 – A nucleocapsid budding through the inner nuclear membrane. The membrane is thickened where it is evaginated. Profiles of this type were rarely observed (X 47,000). Bar = 0.2 μm.

the virus when the inner nuclear membrane was evaginated, but did not see evidence of viral envelopment. Participation of Golgi complex-associated vesicles in the envelopment process was pronounced in the hedgehog. Apparently, this is an event and location common to many CMV infections, since virtually identical observations have been made in mouse,[11,12] rat,[29] and human[21,28] CMV infections.

The sequestration of enveloped virus particles within membrane-bound cytoplasmic vesicles also appears to be a common morphologic manifestation of CMV infection. In the mouse, guinea pig, and shrew, the virus-containing vesicles are relatively enormous, frequently occupying most of the infected cell cytoplasm.[11,12,14,15] However, when specific strains of either guinea pig or human CMV's are grown in tissue culture, only a few enveloped virus particles are observed within much smaller vesicles.[6,30] Whether this difference simply reflects a different stage of the infection or represents a real difference in packaging due to the particular CMV strain is not known. Our observations

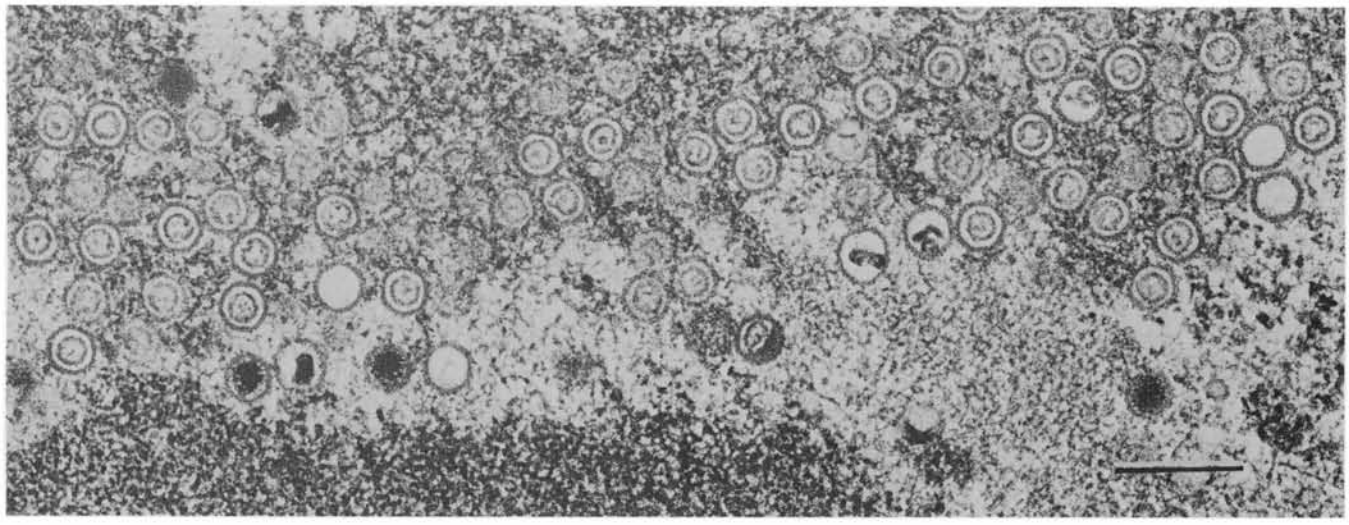

Fig. 5 – Higher power micrograph of the area indicated by arrows in Fig. 4. The majority of the closely packed capsids have a circular nucleoid. A portion of an electron-dense satellite inclusion is in the lower portion of the field (X 87,500). Bar = 0.2 μm.

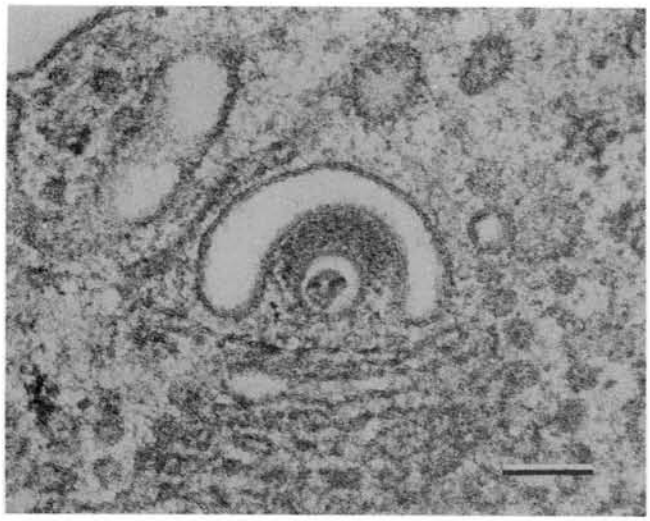

Fig. 7 – A nucleocapsid in the process of envelopment is illustrated. This occurs by indenting vesicles in the Golgi complex (X 66,000). Bar = 0.2 μm.

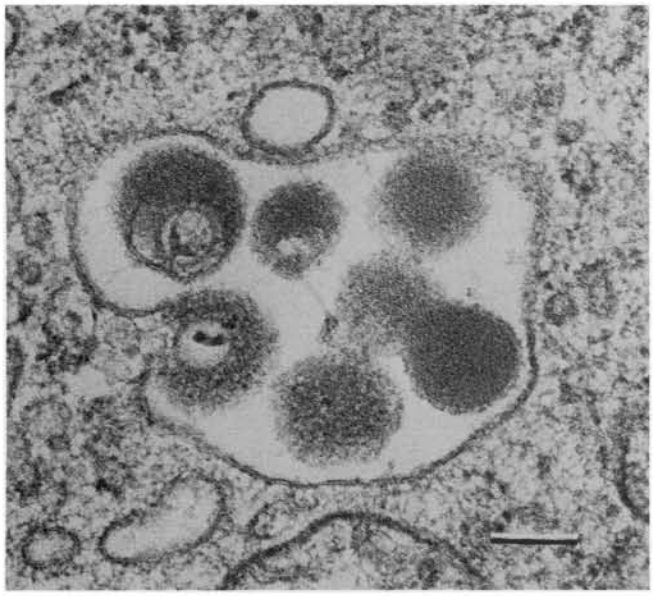

Fig. 8 – An almost completely enveloped virus is surrounded by a condensed matrix except in the region of a slightly constricted cytoplasmic stalk. Presumably the envelopment process will proceed around the nucleocapsid with the particle eventually being released into the vesicular space (X 57,000). Bar = 0.2 μm.

in the hedgehog indicate the virus is sequestered in relatively small numbers in discrete cytoplasmic "granules." Viruses in the hedgehog possessed irregularly-shaped envelopes. In some instances, it is possible that non-viral "microbodies"[30] were sequestered along with the viruses. These latter structures are thought to be aggregates of CMV structural proteins.[31] We also observed some viruses in what appeared to be typical secondary lysosomes, an observation supported by Smith and de Harven,[32] who reported that many enveloped CMV's reside in vacuoles possessing lysosomal enzymes. We were not able to determine the mechanism of viral release into the duct lumen.

The hedgehog submandibular gland has many distinctive cytological characteristics that make it a worthy subject of study for the cytologist interested in secretion.[18,19] Our chance finding of a submandibular gland CMV infection that is typified by cytomegaly, more pronounced than that reported in any other species, justifies further study of naturally-occurring or induced CMV infections in this animal.

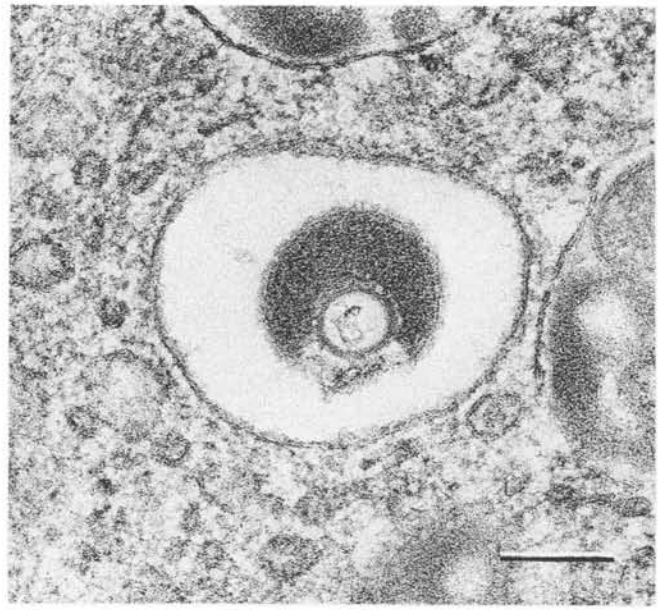

Fig. 9 – An enveloped virus contained within a cytoplasmic vesicle is illustrated. Nucleocapsids usually were located eccentrically within the envelope, a portion of which appears disrupted in this micrograph (X 67,000). Bar = 0.2 μm.

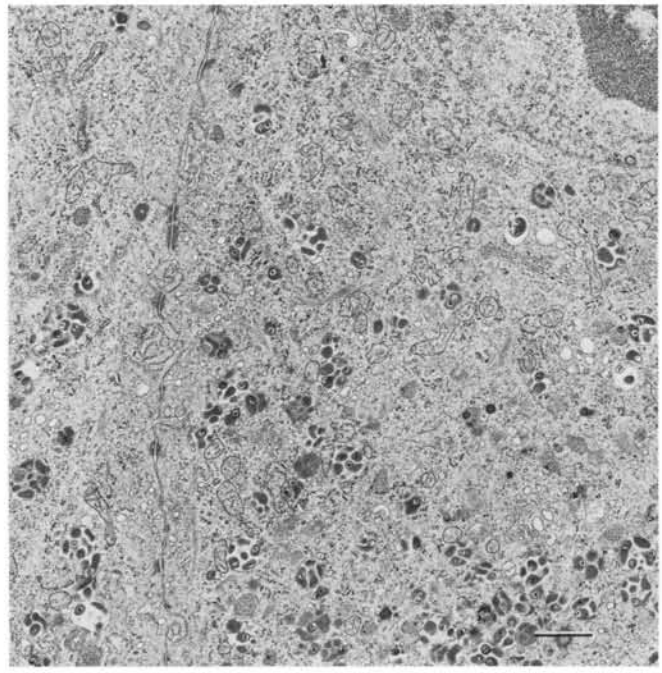

Fig. 10 – Survey micrograph of an infected cell's cytoplasm. The paranuclear Golgi complex encompasses some small virus-containing vesicles, as well as lysosome-like structures. Adjacent to the Golgi zone are numerous virus-filled vesicles that comprise the darkly staining cytoplasmic granules observed with the light microscope. The lateral cell border and a portion of the nucleus are also illustrated (X 14,500). Bar = 1.0 μm.

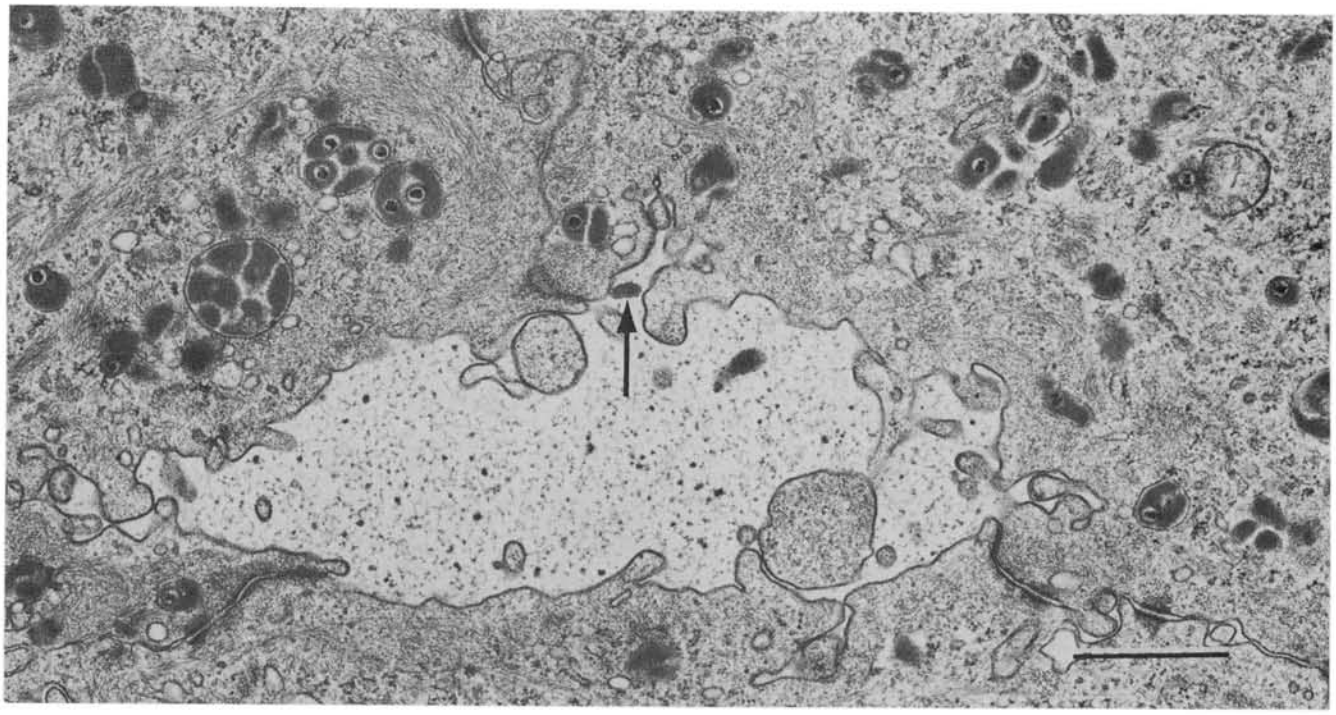

Fig. 11 – The duct lumen is illustrated. The cell apices contain virus-filled vesicles. The mechanism of viral release into the duct lumen was not observed, although solitary viruses (arrow) were present within the lumen (X 19,500). Bar = 1.0 μm.

Conclusions.

Pronounced duct cell gigantism occurred in the submandibular salivary gland of a European hedgehog. The nuclei of the afflicted cells contained honeycomb-shaped or reticulate inclusions that are characteristic of cytomegalovirus infections. Ultrastructural study of the hypertrophied cells revealed numerous viral nucleocapsids associated with the intranuclear inclusions. Nucleocapsids appeared to traverse the nuclear membranes without acquiring an envelope – the latter structures being formed during transit across the membranes of Golgi complex-associated vesicles. Virus-filled vesicles occupied the apical half of the cytoplasm, and occasional viruses were observed within the duct lumen.

Cytomegaloviruses undergo the classical morphologic stages of replication in hedgehog submandibular gland duct cells. A profound cytomegaly is associated with the infection. These features indicate that the hedgehog may be an excellent model in which to study the biology of cytomegalovirus-salivary gland interactions.

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