The Fine Structure of Nuclear and Cytoplasmic Inclusions in Primary Renal Tumors of Mutant Leopard Frogs

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Summary

This investigation summarizes a study of the incidence and fine structure of primary renal tumors found in mutant leopard frogs (Rana pipiens burnsi). Of 204 winter frogs autopsied from Minnesota and South Dakota, renal tumors were found in 13 animals, an incidence of 6.4%. Light microscopic examination showed that all of the renal tumors had Cowdry Type A nuclear inclusions. The fine structure revealed virus and viral-associated structures previously described in inclusion-containing tumors of wild type leopard frogs. Several features were observed which have not been previously described. These were: (a) long, tubular elements associated with nuclear inclusions containing immature and mature virus particles; (b) immature virus particles within the marginated chromatin; (c) images which suggest more than 1 method of viral release from the nucleus; and (d) the migration of mature virus particles into the lumens of agranular cisternae or vacuoles, during which process the virus is enclosed in an extracapsular envelope. This envelope is formed by a pinching off of a portion of the agranular membrane.

Introduction

Lucké (8) first described a renal adenocarcinoma that occurs in leopard frogs (Rana pipiens) from the Lake Champlain region of Vermont and adjacent areas of Canada. The etiology of this tumor has been studied by the following methods: (a) intraocular transplantation, injection of tumor homogenate (9, 10, 29); and (b) injection of cell-free extracts (5). Recently, extracts from primary tumors and urine of infected frogs were inoculated into tissue culture cells (7, 20). These investigations suggested that a virus caused the tumors. Electron microscopic observations of renal tumors from Vermont leopard frogs revealed that some tumors contained virus-like particles (6, 12, 13, 28, 29), but the relationship of these particles to tumor formation has not been established (7, 18–20).

The leopard frog has the largest natural range of any anuran in the western hemisphere. This species is distributed over North America from the Atlantic Coast to the eastern edge of the Pacific Coast States, and from the extreme north into Mexico (27).

The incidence and morphology of leopard-frog renal tumors from other parts of its range have recently been reported. Lungen et al. (13) found 15 primary renal tumors in 930 frogs (R. pipiens) from the Minnesota-Wisconsin area. This was an incidence of 1.6%. McKinnell (15) examined 884 frogs from the same area and found 79 spontaneous renal tumors, an incidence of 8.9%. Steiner (23) suggested that knowledge of racial and geographic distribution of infected animals can illuminate many problems in cancer research. He discussed the importance of racial differences to the incidence and types of cancer. Although most studies of the renal tumors of leopard frogs have been made with animals purchased from the Vermont area, Lynn and Zweifel (14) have emphasized the hazards of assuming that frogs come from this locality since many of the dealers obtain frogs in wholesale quantities from distant areas.

Though the range of R. p. burnsi is large, the distribution of its genetic variants is limited and well known. One of the variants described as a new species (26) is R. pipiens burnsi which is easily recognized by the lack of dorsal body spots. Subsequent genetic studies have shown that it differs from the common leopard frog by a single dominant gene (17, 25). The use of burnsi frogs which have renal tumors permits the study of a different strain from limited areas of South Dakota and Minnesota (16).

Spontaneous renal tumors were found in a few leopard frogs from North Dakota, Indiana, and Mississippi Valley (10). Rafferty (19) suggested in a recent review that tumor-bearing frogs occur in Minnesota and eastern South Dakota; this prediction has recently been confirmed (4, 13, 15, 30).

This investigation describes the fine structure of virus particles found in 13 spontaneous renal tumors of burnsi mutant leopard frogs.

Materials and Methods

SOURCE OF TUMORS. R. p. burnsi mutants were obtained January 2, 1964, from J. R. Schettle Frog Farm, Inc., Stillwater, Minn. The frogs were maintained in squat 2-qt glass bowls with pond water in a refrigerator (4°C). The frogs were killed by brain pithing, and the snout-vent length was recorded prior to autopsy (males 45–57 mm and females 59–93 mm). Both dorsal and ventral aspects of the kidneys were examined for abnormalities.

LIGHT MICROSCOPY. Tumor tissue was fixed in Bouin's solution;
8-10-µm sections were stained with Heidenhain's iron hematoxylin and eosin. These specimens were used for preliminary evaluation and comparison of materials used in electron microscopy with observations by earlier authors.

**Electron Microscopy.** Small fragments of tumor tissue were fixed for 1 hr in 4% glutaraldehyde buffered with 0.2 M s-collidine (pH 7.6) at 4°C, and then fixed for 1 hr in 2% osmium tetroxide buffered with 0.2 M s-collidine (pH 7.6). The tissue was rapidly dehydrated through a graded ethanol series, and embedded in Epon S12 (11). Thin sections were cut on Porter-Blum microtomes, models MT-1 or MT-2, and placed on uncoated 75 x 300 or 200-mesh copper grids. The sections were double-stained, using a 2% aqueous solution of uranyl acetate for 10 min at 60°C followed by lead citrate (22) for 5 min. The sections were examined with an RCA EMU-2D, EMU 3-F, or Philips EM 200 electron microscope.

**Results**

**Incidence of Tumors.** Two hundred four burnsi frogs were examined, 87 males and 117 females. Primary renal tumors were found in 13 animals, an incidence of 6.4%; of these 5 were from males, 5.7%; and 8 were from females, 6.8%. Nine of the animals contained unilateral tumors, and the remainder were bilateral. The tumors varied from 1 to 6 mm in diameter. No relationship between size of the host animal to tumor size was noted.

Light microscopic morphology of these tumors corresponded to previous descriptions (5, 6, 8, 10, 24).

Electron microscopic examination of the primary renal tumors showed nuclear and cytoplasmic inclusions. The chromatin of nuclei of tumor cells with Type A inclusions was usually marginated (Figs. 1, 2). A fine, opaque, granular material was dispersed within the nucleus. This substance occurred in small aggregates, in filament-like structures, and in clumps up to 400 µm in diameter (Figs. 1, 3, 4, 6).

The nuclear inclusions were composed mainly of single-membraned capsids (90-100 µm in diameter) which lacked nucleoids. They were scattered through the nucleoplasm (Figs. 1, 2, 7, 8, 13), often close to and occasionally within the marginated chromatin (Fig. 2). Frequently, these particles were found densely packed and arranged in crystalline arrays (Figs. 1, 8). Although the majority of the single-membraned capsids were empty, a few contained nucleoids (Figs. 1-3, 13). Other types of particles in nuclear inclusions were: (a) free nucleoid-like bodies 40-60 µm in diameter often associated with empty single-membraned capsids (Figs. 1, 2); (b) double-membraned capsids without nucleoids (Figs. 1, 4, 5); (c) double-membraned capsids that are 115-135 µm in diameter with nucleoids (Figs. 7-9). The virus particles were usually enclosed in a membranous sac near the inner surface of the nucleus, often causing the nuclear membrane to bulge (Fig. 9).

In addition to the above-mentioned nuclear inclusions, tubular elements have been observed in over 50% of the burnsi tumors examined in this study. They may occur singly or in groups and depending on the plane of section may be seen as masses of entwined tubules (Figs. 3, 13) or densely packed rows of tubules in either longitudinal or cross-sectional view (Fig. 4). The diameter of the tubular elements varies from 50 to 80 µm, and their length, depending upon the plane of section, up to 8-10 µ (Figs. 6, 7). The cross-sectional morphology of these elements was similar to empty double-membraned capsids.

Cytoplasmic virus particles were composed of double-membraned capsids with nucleoids. They may be scattered throughout the cytoplasm or found in clusters. It was not unusual to find a single virus or a cluster of viruses enclosed within agranular membranes (Figs. 10-13). The virus particles within these membranes had acquired an additional extracapsular envelope which was the same thickness as the surrounding cytoplasmic membrane (Figs. 10-12). This extracapsular envelope had not been observed around the nuclear virus particles which were segregated from the nucleoplasm by a membrane.

Frequently associated with cytoplasmic virus particles were bundles of dense filaments (Figs. 11, 13), varying from 4-8 µm in length. The filaments were described by Fawcett (6). Their origin and function is still obscure; however, mature virus particles were frequently seen in intimate association with them. The capsids of these virus particles had become thickened and granular, losing their crisp outline (Figs. 11, 13). Another type of cytoplasmic inclusion described by Fawcett (6) is vacuolar aggregations (6). These vacuoles (Figs. 10, 11) varied from 1 to 5 µm in diameter and, frequently containing virus particles, were often associated with the Golgi complex.

Within the renal tumor tubules, extracellular virus particles were frequently seen. They had double-membraned capsids with nucleoids and an additional extracapsular envelope, and varied from 115 to 135 µm in diameter. Rarely did one see incomplete virus particles in extracellular spaces unless they were associated with cells undergoing autolysis. These extracellular particles contained a fine granular substance which was observed between the inner surface of the extracapsular envelope and the outer surface of the capsid. Frequently, this substance fills this space (12, 13, 29) (Fig. 14).

**Discussion**

The present study based on 204 burnsi frogs, with a primary tumor incidence of 6.4%, suggests that the tumor may occur at a higher frequency in the North Central United States than in the Vermont area.

Rafferty (19) postulates that prolonged low temperature may be the principal factor favoring nuclear inclusion formation and virus production. The burnsi frogs used in this study were obtained in winter. All of the renal tumors examined contained virus particles. Rafferty's (19) contention that virus production and low temperature are related has been supported by a monthly study of frogs (Zambernard, unpublished results.)

The fine structure of renal tumors in burnsi frogs correspond to the previous descriptions (1, 6, 29) of renal tumors of Vermont leopard frogs. Consequently, the discussion will be restricted to virus particles and virus-associated structures found in these tumors. The probable sequence of maturation for the leopard frog virus has been reconstructed from static images (6, 13). This paper will consider those structures not previously reported and a possible sequence of events.

Fawcett (6) proposed that the aggregations of dense granules found in the nucleus of virus-containing cells condense to form the viral nucleic acid core (nucleoid) which migrates into the empty single-membraned capsids to give rise to an immature virus...
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(i.e., single-capsid and nucleoid) (Figs. 1, 2, 13). Although the opacity and texture of the large, granular clumps (100-300 mµ) are similar to naked nucleoid particles, empty single-membraned capsids are rarely seen in their proximity. An alternative function of these clumps might be the production of capsid material. The granular clumps (Fig. 2) are actually composed of different-sized capsids which appear to be in various formative stages. Techniques are now available for direct studies of the chemical constitution of these dense bodies, for example (a) biosynthetic inhibitors, (b) radio-labeled amino acids and nucleic acids, (c) electron microscopic autoradiography, and (d) enzymes (30).

Single-membraned capsids have been reported adjacent to marginated chromatin (13). We have observed both empty single-membraned capsids as well as those with nucleoids, not only in close proximity but within the marginated chromatin (Fig. 2). Since the virus is known to contain DNA, the location of these immature particles in the chromatin might be significant, particularly with respect to the mode of nucleoid acquisition.

An interesting nuclear inclusion is the tubular elements. These structures do not occur in all inclusion-containing renal tumors, and when they do occur they vary considerably in length and number. They were not observed in the renal tumor cells examined by Fawcett (6). Lunger et al. (13) reported the presence of very short tubular elements in tumors and believed that these elements do not fragment to form double-membraned capsids. The function of these tubular elements is speculative. We have found that these tubular elements are digested by pepsin but not by trypsin (Zambernard, unpublished observations). We have seen both empty single- and double-membraned capsids (Figs. 3-7, 13) as well as single- and double-membraned capsids with nucleoids (Figs. 3, 4, 6, 7, 13) in close association with the tubular elements. When this occurs the capsids appear granular, become slightly thickened, and lose their clear outline (Fig. 5). A similar observation was reported (13) for virus particles associated with dense cytoplasmic filaments (Figs. 11, 13). aberrant forms similar to the tubular elements observed in leopard-frog renal tumors have been observed and described from partially-purified papilloma virus preparations (2). Since the DNA of the papilloma virus does not appear to be enclosed by the tubular forms, the authors suggested these tubular elements represent mistakes in subunit assembly.

Lunger et al. (13) suggest that the empty double-membraned capsids play an important role in the developmental cycle of the virus. They point out the possibility that inner ring of the double-membraned capsid undergoes condensation or coalescence to form the nucleoid. Recent enzyme experiments (30) have shown that empty capsids (single- and double-membraned) are hydrolyzed by 0.5% solution of pepsin in 30 min. Our data indicates the inner membrane of empty double-membraned particles is protein. Casper and Klug (3) suggest that the subunits of empty capsids found associated with most icosahedral viruses are assembled without the need of a nucleoid or any external organizer. The evidence for the self-assembly of icosahedral capsids was provided by adding 5-fluorouracil to cells infected with a pseudorabies virus. Since 5-fluorouracil blocks DNA synthesis the particles that were produced appeared the same morphologically as the intact infectious virus, except they lacked nucleoids. The evidence from such an experiment indicates that the capsids were produced by a spontaneous aggregation of the protein subunits in the infected cell (21). They suggest that the nucleic acid is packaged in preformed capsids.

Within a few of the renal tumor nuclei, we have seen particles which have double-membraned capsids with nucleoids. Fawcett (6) interpreted these to be the mature infectious particle. When seen in nuclei, these particles are always found within a membranous sac (Figs. 7-9). Fawcett speculated that the virus particles migrated from the nuclei into the cytoplasm by dissolution of the nuclear membrane. Fig. 8 shows there is a complex relationship existing between the viruses, which are in a membranous sac, and the nuclear membrane. The nuclear membrane appears to rupture in this area allowing the particles to enter the cytoplasm. When this occurs, there is virtually no loss of nuclear content since the lateral portions of the membranous sac fuse to the inner nuclear membrane, making it continuous (Fig. 8). It appears that mature viral particles can be discharged from the nuclei individually rather than all at once. This occurs as a budding-off process (Fig. 9).

Once in the cytoplasm, the virus particles may be scattered (Figs. 11, 13) or in clusters (Fig. 10). Frequently, they are intimately associated with dense proteinaceous fibers (Zambernard, unpublished observations) as described by Fawcett (6). Lunger et al. (13) mention that capsids of virus particles become thickened and fuzzy when they are associated with dense fibers (Figs. 11, 13). The significance of these fibers in viral reproduction is still a matter of speculation.

Of importance is the manner in which these mature virus particles acquire an additional extracapsular envelope. Fawcett (6) stated that he believed the extracapsular envelope was acquired from the host cell membrane as the virus particle was discharged into the lumen of the tumor tube. Lunger et al. (13) found that the virus particles acquired an extracapsular envelope while still in the cytoplasm, in the region of the Golgi complex where a flattened cisterna gradually surrounded the particle, resulting in a double-membraned particle with an extracapsular envelope. We have not observed this in our study. Virus particles were observed in what appeared to be enlarged vesicles of the Golgi or in enlarged agranular cisternae. The virus particle migrates into these vesicles carrying part of the vesicle membrane along with it. As the virus migrates deeper into the vesicle the membrane gradually closes off behind it (Figs. 10-12). The final step is a pinching-off process which results in the acquisition of an extracapsular envelope by the virus particle (Figs. 11, 12) which is now entrapped within an enlarged vesicle. Fig. 10 shows a large vacuole in the cytoplasm packed with virus particles. The particles all have extracapsular envelopes. This vacuole appears to be part of an enlarged Golgi vesicle. Infrequently, mature virus particles are found within vesicles in the cytoplasm without the extracapsular envelope (Fig. 13).

Mature extracellular virus particles observed in the lumens of tumor tubules are enclosed in an extracapsular envelope (Fig. 14). Fig. 15 is an enlarged extracellular virus particle and clearly demonstrates the morphologic components (i.e., nucleoid, double-membraned capsid, and extracapsular envelope). Thus far we have not observed the method by which the virus particles are discharged into the lumens of the tumor tubules. Fawcett (6) speculated the process may involve a budding off from the cell surface of minute vesicles containing individual virus particles. Although virus particles have been observed close to the inner...
and outer free surface of tumor cells, this mechanism of viral release has not been seen in the tumors we have examined.

It should be kept in mind that the great variety of structures described in conjunction with these inclusion-containing tumors may also be an expression of more than 1 type of virus or errors in viral assembly. On the basis of fine structure the virus particles are identical; for this reason it is necessary to isolate and study the viruses by immunologic methods.

Although viruses are found in leopard frog renal tumor cells, only further experimentation will show whether or not they are the causative agent of the tumor.

Finally, we would like to mention that up to now we have not observed virus particles in the lumens of proximal, convoluted tubules of normal kidney cells (13). The well-developed microvilli at the free surface of these renal tumor cells could lead one to this misinterpretation (Fig. 14). Fawcett (6) suggested that the tumor originated in the proximal tubule of the kidney. One of the criteria for this was based on the well-developed microvilli at the free surface of the tumor cells. However, the microvilli of tumor cells are never as compact or as uniform in height as those found at the free surfaces of normal proximal tubule cells. Lücke (10) was aware of the different gradations of malignancy in these tumors and stated, "All gradations are found between the frankly malignant, invasive and destructive adenocarcinoma to the structurally benign adenoma, cystadenoma and papillary cystadenoma." We must remember that cells in the process of undergoing tumorigenesis will resemble their normal counterparts more closely than the frankly malignant cells. We have found that, morphologically, the inclusion-containing renal tumor cells of the leopard frog resemble their normal counterparts more closely than renal tumor cells which lack these inclusion bodies.

References

FIG. 1. A longitudinal section through a tumor nucleus containing Type A inclusion (arrows). Marginated chromatin (Chr) as well as scattered clumps of dense granules (DG). These clumps seem to be composed of aggregates of granules and short filaments (FG) scattered throughout the nucleoplasm. The assortment of virus-associated particles seen within this nucleus are: a crystalline array of particles composed primarily of single-membraned capsids without nucleoids (A), a few single-membraned capsids with nucleoids (B), double-membraned capsids without nucleoids (C), and several naked, nucleoid-like bodies (D). Within this nucleus are readily visible nucleoid-like bodies which appear to be in the process of being enclosed in single membraned capsids (E). \( \times 36,800. \)

FIG. 2. Tumor nucleus showing single-membraned capsids without nucleoids (A) and with nucleoids (B) in close proximity as well as within the marginalized chromatin (Chr). Visible also are many nucleoid-like bodies in what appears to be various stages of encapsulation. Nucleoids next to empty single-membraned capsid (a), nucleoid beginning to enter capsid (b), nucleoid almost within capsid (c), and nucleoid within capsid (d). Clusters of single-membraned capsids (Ca) of varied sizes appear to be in formative stages. \( \times 26,300. \)

FIG. 3. Entwined tubular elements (TE) of varying lengths are intermingled with various stages of immature virus particles. This is a portion of an intranuclear inclusion. \( \times 40,000. \)

FIG. 4. Parallel arrays of tubular elements (TE) in longitudinal section within the nucleus. Single-membraned (A) and double-membraned (B) capsids are found in close association to these structures. Also visible are double-membraned capsids with a small inner diameter (C), these are tubular elements in cross-section. Note the difference in the morphology of structures labeled B and C. \( \times 38,000. \)

FIG. 5. A parallel array of tubular elements (TE) surrounded by empty double-membraned capsids (arrows). Note that the inner diameter of tubular elements is smaller than the inner diameter of the double-membraned capsids. \( \times 123,000. \)

FIG. 6. The nucleoplasm of this tumor nucleus contains scattered clumps of dense granules (DG) and a longitudinal section through several tubular elements (TE). Note the length of the tubular elements and their intimate relationship with immature viral particles (arrows). \( \times 21,000. \)

FIG. 7. Within this tumor nucleus are seen numerous, single-membraned capsids (A) without nucleoids. Several longitudinally-sectioned tubular elements (TE) are in intimate relationship with mature virus particles (VP). Note that the mature particles and tubular elements are enclosed within a membranous sac (MS). \( \times 24,000. \)

FIG. 8. Several crystalline arrays composed mainly of single-membraned capsids are seen within this nucleus (A). Note that the membranous sac (MS) containing mature virus particles (VP) appears to have ruptured along with the nuclear membrane, thus allowing the contents of membranous sac to be emptied into the cytoplasm (arrows). \( \times 24,000. \)

FIG. 9. Another membranous sac containing mature virus particles can be seen in this nucleus. Note how the sac has caused nuclear membrane to protrude into cytoplasm (arrows). Visible is a mature virus particle which appears to be budding off from the nuclear membrane (A). This may be another method by which mature nuclear viruses may be released into cytoplasm. \( \times 46,000. \)

FIG. 10. An enlarged cytoplasmic vacuole packed with mature virus particles. All of the viruses within this vacuole have acquired an extracapsular envelope (A). One particle (B) appears to be still in the process of migrating into this vacuolar sac. Note that this vacuole is in close proximity to the Golgi complex (GC) from which it may have arisen. Also there are several dilated agranular cisternae (arrows) which may also be part of the Golgi complex. \( \times 34,000. \)

FIG. 11. Within this vacuolar aggregation (V) several virus particles are in the process of completing their migration into the cisternae (A). Note that they are still attached to the vacuolar membrane. Another virus particle (B) is in contact with a vacuolar membrane and appears to be just beginning its migration into the vacuole. Also visible is an aggregation of dense fibers (DF) in cross and oblique section; notice that there are many mature virus particles (VP) in close association to these fibers. The capsids of these viruses are thickened and granular. \( \times 54,000. \)

FIG. 12. Four mature virus particles within a cytoplasmic agranular vesicle. Note that 3 of the virus particles are still in the process of completing their migration into the vesicle. The membrane of the vesicle appears to be in the process of pinching off behind the particles (arrows). This shows 1 process by which virus particles may acquire an extracapsular envelope. \( \times 54,000. \)

FIG. 13. Part of a tumor nucleus is seen in the lower left hand corner of this micrograph. Within the nucleoplasm are entwined tubular elements (TE) of varying sizes. Single-membraned capsids without nucleoids (A) and with nucleoids (B) make up the majority of immature particles. Two single-membraned capsids appear to be in the process of acquiring nucleoids (C). The prominent cytoplasmic structure in a supranuclear position is a mass of dense fibers (DF) in longitudinal section. Note the length of some of these fibers. Frequently associated with these fibers are virus particles (arrows). The capsids of these particles become thickened and acquire a more granular texture. Visible also are agranular vacuoles of various sizes. Notice that 2 of these vacuoles contain virus particles without an extracapsular envelope (D) whereas in another vacuole a virus particle has the extracapsular envelope (E). \( \times 40,000. \)

FIG. 14. Extracellular virus particles within the lumen (L) of a tumor tubule. All of these viruses have an extracapsular envelope (arrows). Notice the prominent microvilli at the free surface of these renal tumor cells (MV), they lack the uniform arrangement of microvilli found at the free surface of normal cells of the proximal tubule of the frog kidney. \( \times 46,000. \)

FIG. 15. The fine structure of an extracellular virus particle. Note the prominent nucleoid (Nuc), the double-membraned capsid (C1 + C2), and the extracapsular envelope (EE). \( \times 170,000. \)
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