Herpesviruses in Tumors of Postspawning *Rana pipiens*  

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**SUMMARY**

Herpesvirus particles were observed in renal tumors of *Rana pipiens* collected in rural Minnesota as long as 42 days after spawning. Tumor cytology differs from tumors of frogs collected during cold weather and suggests that the tumors are in transition from the virus particle-containing state to the inclusion-free state. The presence of viruses in tumors of frogs in breeding ponds may have epidemiological significance.

**INTRODUCTION**

The demonstration of viral DNA in cells of most virus-induced tumor systems remains a technically formidable problem (5). The renal adenocarcinoma of the North American leopard frog, *R. pipiens*, provides a notable exception to this generalization. Indeed, over 30 years ago a virus was thought to be the etiological agent of this tumor because of the presence of Cowdry type A inclusions, which can be detected by light microscopy (7). Herpesvirus particles were revealed subsequently by direct observation with the electron microscope (2, 8, 26). Mature virus particles are invariably present in algid (cold weather) and transitional (postspawning) renal tumors (9). The only frog renal tumor occurring in nature that is devoid of virus particles is the tumor found prior to the time at which frogs enter lakes for the cold season (23).

Transmission of the Lucké herpesvirus is not understood (22). The present study describes the cytology of tumors and the presence of viruses 52 days after emergence of frogs from lakes in the spring and 42 days after fertilization of eggs. We believe that the presence of viruses in postspawning *R. pipiens* provides information that may be useful in establishing the mode of transmission of this suspected oncogenic agent. Our observations are consistent with, but do not establish, the hypothesis that contagion occurs in breeding ponds.

**MATERIALS AND METHODS**

**Tumor Frogs.** Collections of frogs were made in breeding ponds of central and western Minnesota at about weekly intervals during the spring of 1971. Although mature frogs overwinter in bodies of water that do not freeze solid during the cold season, such as deep-water lakes, they move to shallow breeding ponds after the ice melts on the lakes.

On April 13, 1971, frogs were observed on the margins of overwintering lakes and in areas between the lakes and breeding ponds in Kandiyohi County, 100 miles west of Minneapolis, Minn. No frogs were observed in breeding ponds on that day. Frogs were occupying breeding ponds in the area by April 16, 1971, but a search for egg masses revealed none. The same breeding ponds were scrutinized again on April 23, 1971. Many male frogs were calling, numerous frogs were in amplexus, and about 20 large egg masses were observed in a small area of 1 pond. By May 4, 1971, swimming tadpoles had hatched from the egg masses. Virus-containing tumors collected from this locality earlier in the season were described elsewhere (9). Three tumors were detected by autopsy of frogs collected from Kandiyohi County on June 3, 1971. These renal adenocarcinoma-bearing frogs were collected from breeding ponds 5.5 weeks after spawning. Tadpoles occupied the pond with the adult tumor-bearing frogs.

Three other renal adenocarcinomas were obtained from frogs found in breeding ponds in Otter Tail County, Minn., about 175 miles northwest of Minneapolis on May 12, 1971. The time of spawning is not known for the Otter Tail County collection, but it was probably several weeks prior to the collection date, as judged by the size of the tadpoles in the breeding ponds.

**Electron Microscopy.** Frogs were autopsied in Minneapolis within 24 hr of capture in the field. Tumor fragments of about 1 cu mm were fixed for 1 hr in cold 4% glutaraldehyde buffered with s-collidine (pH 7.3 to 7.6). Fixed tissue was rinsed and stored in s-collidine buffer. Cold 2% osmium tetroxide buffered with s-collidine (pH 7.3 to 7.6) was used for 1 hr postfixation. The tumor tissue was dehydrated with ethanol and propylene oxide and subsequently embedded in Epon. The tissue was sectioned with a Reichert OM U2 ultramicrotome and placed on Parlodion-coated grids. The sections were stained with 2% aqueous uranyl acetate and 1% lead citrate and examined with an AEI EM 801 electron microscope.

**RESULTS**

All 6 renal adenocarcinomas of postspawning *R. pipiens* contained mature herpesviruses. The lumina of the tumor tubules contained sloughed tumor cells in varying degrees of degeneration. These cells, which were undergoing lysis, as evidenced by membrane and organelle breakdown, contained viruses in various stages of maturation (Fig. 1). Although
Many cells also had cytoplasm free of viruses detectable with enveloped immature viruses were found in the lumina. In most cases, the capsid was located eccentrically within the envelope (Figs. 1 and 2). No unidentifiable cellular debris. Mature virions contained an intact nuclei and other cellular organdíes could be seen in some sloughed tumor cells, mature infectious virions, which were liberated into the lumina, were present in large numbers. Viruses lacking DNA cores were present in the lumina in and some sloughed tumor cells, mature infectious virions, which were liberated into the lumina, were present in large numbers. Intact nuclei and other cellular organelles could be seen in some lumina, but generally the lumina were packed with unidentifiable cellular debris. Mature virions contained an electron-dense material between the virus capsid and the extracapsular envelope. In most cases, the capsid was located eccentrically within the envelope (Figs. 1 and 2). No enveloped immature viruses were found in the lumina.

No nuclear inclusions were seen in any intact tubule cell. Many cells also had cytoplasm free of viruses detectable with the electron microscope. However, these virus particle-free cells were in close proximity to mature enveloped virions present in the tubule lumina (Fig. 3). Some tubule cells contained viruses, but the particles were found within cytoplasmic vacuoles that contained cellular debris as well as mature viruses (Fig. 4).

We have no record of daily air temperature at Diamond Lake in Kandiyohi County, but we do have the daily minimal and maximal temperatures for Willmar State Hospital (Chart 1), located approximately 10 miles west of Diamond Lake. The temperatures are for that period of 1971 from the time of emergence from overwintering through June 3, 1971, which was the date of the last collection of tumor frogs. Viruses persisted in the tumors throughout the entire period, despite 19 days in which the air temperature exceeded 21° (a temperature that will bring about a rapid change to the calid state in the laboratory) (25). However, on 32 days (Chart 1), the minimal temperature fell below 7.5°, a temperature that will cause the appearance of viruses in “virus-free” tumors in the laboratory (20).

DISCUSSION

The Ubiquitous Lucké Tumor Herpesvirus. No special techniques are required to detect the Lucké tumor herpesvirus in spontaneous tumors of field-collected frogs obtained from early hibernation through a period well beyond spawning. In an earlier study, 11 renal tumors were obtained from frogs that were emerging from overwintering in lakes. Each of the 11 contained virus particles detected by electron microscopy (13). Subsequently, viruses occurring in 18 consecutive renal tumors that were taken from frogs in hibernation were described (9). The present study concerns virus particles in each of 6 renal tumors obtained from frogs found in breeding ponds. These studies comprise a total of 35 spontaneous renal tumors, each with herpesviruses. There were no exceptional (i.e., virus-free) renal tumors. The invariable presence of herpesviruses in tumors of overwintering and reproductive frogs does not seem to us to be a chance relationship.

The only frog renal tumors from natural populations that are devoid of virus particles are the calid tumors obtained from frogs prior to overwintering (23). If one may extrapolate from laboratory studies of the effect of cold temperature on virus replication (1, 15, 16, 19, 20), one would prognosticate that the calid, or virus-free, tumors would shortly be in a virus-replicating phase because the water temperature of the overwintering lakes is cold. Our field observations show that mature viruses are present in some tumors within 7 days after the onset of hibernation (10).

Tumors from Overwintering and Postspawning Frogs Compared. What is the difference between a virus-containing algid tumor and a virus-containing transitional tumor? Spontaneous algid tumors derived from natural populations do not seem to differ from the virus-containing tumors that have been described elsewhere (2, 24, 26). In transitional tumors, cells that are still attached to the basement membrane have nuclei that are devoid of virus particles. The lack of virus particles suggests that the tumor is in transition from a stage of active virus production to a stage of no virus production. Many cells that contain virus particles appear to have detached from the epithelium and, because of their poor cytoplasmic structure, seem to be in the process of lysis. This process is similar to loss of nuclear inclusions and induced lysis of virus-containing tumor cells in frogs by elevated temperature (16, 25), but one difference is striking and may be of epidemiological significance. The difference is that, while lysis occurs in the laboratory after 3 to 5 days at 20 to 22°, lysis is still incomplete 52 days after emergence of the host frog from hibernation.

Although detached cells contain viruses, detachment from the tubule epithelium is not a prerequisite for virus replication. Viruses are observed in attached cells within 1 week of the onset of hibernation in the autumn (10), but the transition from the algid tumor type (with viral inclusions) to the inclusion-free calid tumor type does not occur until approximately 6 months later.
The Natural History of the Frog and Possible Routes of Transmission of an Oncogenic Agent. The principle of the conservation of energy would suggest that it would be imprudent of an etiological agent to be produced and disseminated at a time when there is neither a route of contagion nor a susceptible host available. One may gain insight into the mode of infection of an oncogenic agent by correlating agent production with susceptible stages in the life cycle of the host. A fruitful hypothesis may emerge if agent production and susceptible stage are synchronous.

Eggs and early embryos are susceptible to tumor induction by a tumor brei containing mature herpesviruses (12, 21, 22). The herpesvirus of the Lücké tumor thus far is known only from intact tumor cells, from fluid contained in the lumina of tumors, or from urine of tumor-bearing frogs (3, 18). We know from the present study that tumor-bearing frogs contain viruses for a prolonged time in the breeding pond. Thus, it would seem reasonable to suggest that transmission occurs in the breeding ponds. We are not the first to suggest this possibility (17, 18); however, the present study provides the first information as to the duration of time that frogs with tumors may be releasing viruses to the environment.

After the breeding season, juvenile and adult frogs migrate away from the breeding ponds (14). Large adults become more or less solitary individuals and frequently inhabit a moderately xeric environment. From what is presently known concerning the frog tumor virus, it seems unlikely that it can be passed from individual to individual if a water route is absent. Tumors are scarce during the warm summer months (11), and the few that are found appear to be devoid of virus particles (23). Adults seem to be more immune to induction of tumors by viruses than immature or neonatal vertebrates (4, 6), and the frog does not seem to be an exception to this generalization (12, 21, 22). Accordingly, we would like to suggest that transmission does not occur during the warm months of the year when frogs with calid tumors are foraging in meadow and field.

With the onset of cold weather, frogs migrate to overwintering lakes. Shortly after the frogs enter the lakes, virus particles may be observed (10). Crowding may be considerable at the bottom of lakes or streams during the boreal Minnesota winter (14). Algid tumors have mature viruses, which are probably released into the lake water (3, 18). We considered the probably minimal susceptibility of adults to virus infection and tumorigenesis above. Accordingly, we deem improbable the hypothesis of horizontal transmission of the herpesvirus during overwintering on the grounds that a susceptible phase of the life cycle of the host is lacking.

We favor the hypothesis of transmission in breeding ponds. Susceptible stages (eggs and embryos), mature viruses, crowded frogs, and a water route that may connect the host and the agent are all present. Such a hypothesis, should it prove to be true, does not exclude the possibility of vertical transmission from a tumor-bearing, sexually mature adult to its offspring via gamete infection.

How Temperature Sensitive is a Virus-containing Tumor Cell? Lücké (7) wrote that summer and autumn tumors were of the noninclusion variety. Tumors of frogs kept in the laboratory at 20 to 25° were mitotically active and were of the noninclusion type and therefore were probably free of virus particles (19). More recently, the conversion of virus-containing tumors to the calid type within 7 days in the laboratory at 20 to 22° has been shown (16, 25). The virus-containing transitional tumors of this study endured 19 days when the maximal temperature exceeded 21°, but there were 32 days when the minimal temperature was less than 7.5° (Chart 1). It would seem that high temperature is inadequate to cause lysis of cells and loss of viruses if the high temperature is intermittent.

The present study suggests that a reaction such as lysis and release of viruses from tumor cells, which can occur very rapidly in the laboratory, may not occur nearly so rapidly in nature. We suggest that the slower natural rate of virus release may have epidemiological significance.

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REFERENCES

Fig. 1. Cells containing incomplete viruses and cellular debris in lumen of a transitional tumor collected in Kandiyohi County, Minn., June 3, 1971. Arrows, mature viruses in lumen. X 10,400.

Fig. 2. Mature viruses in lumen of a transitional tumor collected in Kandiyohi County, Minn., June 3, 1971. X 14,400.
Fig. 3. Intact tubule cells free of virus particles. Note mature viruses in lumen (arrow). From a transitional tumor collected in Kandiyohi County, Minn., June 3, 1971. X 12,800.

Fig. 4. Cytoplasmic vacuoles containing mature viruses (arrows) in tubule cells from a transitional tumor collected in Kandiyohi County, Minn., June 3, 1971. X 12,400.
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