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Two Viruses from the Lucké Tumor Isolated in a Frog Pronephric Cell Line[1] (37981)

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Since the original proposal of Lucké (1) for a viral etiology of the renal adenocarcinoma of *Rana pipiens* numerous attempts to cultivate the oncogenic virus have led to the isolation of a polyhedral cytoplasmic DNA virus, FV-3, (2, 3), a herpes virus, FV-4, (4, 5) and a papova-like virus (6). An adeno virus, FAV-1, has also been recovered from a granulomatous frog kidney (7).

Bioassay of the Lucké tumor cell fractions has indicated that the oncogenic virus has a preferential affinity for differentiating kidney tissue *in vivo* (8, 9). In the present study, a cell line of differentiating cells was established with pronephric kidneys from larvae of the leopard frog, *Rana pipiens*. The nuclear- and microsomal-viral fractions of a nuclear inclusion Lucké tumor were inoculated into this cell line. An adeno-like virus and the herpes virus were recovered after infection and their combined effect was observed in frog embryos.

Materials and Methods. Donor animals. Larvae of *Rana pipiens*, Shumway stage 24–25 (10) were used as donors for the pronephroi. The larvae were reared in filter sterilized biologically conditioned water with antibiotics (100 U penicillin, 100 μg streptomycin/ml) for 24 hr before harvesting.

Primary pronephric cultures. One hundred pairs of pronephroi were harvested aseptically and washed 5 times in sterile amphibian Ringers solution with antibiotics (1000 U penicillin and 1000 μg streptomycin/ml). From 25–30 pronephroi were seeded into each 75 cm^2 plastic flask and covered with modified Leibovitz-15 medium (11) containing 10% fetal calf serum and incubated at 25°. The cell line subsequently established was designated WMPa, abbreviation for Wisconsin-Minnesota (*R. pipiens*) Pronephros, strain a.

Cell subcultivation. The pronephric cells were subcultured with 5 ml of 0.02% ethylenediamine tetracetic acid plus 0.1 ml of stock Protease (5 mg/ml, Type VI, Sigma Chemical). Dissociated cells were resuspended in L-15 medium with 10% fetal calf serum; about 5×10^4 cells/ml was the optimum concentration. A 1:1 subcultivation was made for the first few subcultures until the cell strain vigor was determined. In later passages a 1:3 or 1:4 subcultivation was made.

Growth rate study. The population doubling time was determined from hemocytometer counts of triplicate 75 cm^2 flasks seeded with 2×10^5 cells/10 ml of L-15. The plating efficiency was based upon the number of cells attached 5 hr after plating.

Tissue culture chromosomes. Actively growing cells were incubated with colchicine (5.3 μl/ml) for 17 hr. The cells were subjected to hypotonic shock with glass distilled water for 10 min, then fixed in acetic acid-ethanol (3:1) for 10 min. They were then stained with Giemsa and the chromosomes of 150–200 metaphase figures were counted.

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Tumor fractions. A tumor bearing frog, RT 208 (*Rana pipiens* Vermont) was kept at 9° for 2½ months and the tumor was removed aseptically. The tumor was first fractionated as described (8) by differential centrifugation into a nuclear fraction (P_1), a mitochondrial-viral fraction (P_2), a mitochondrial supernatant, S_2 and a microsomal viral pellet, P_3. The original nuclear fraction was then sonicated and recentrifuged to yield a sonicated nuclear pellet, PN_{1s}. The remaining nuclear supernatant, SN_1 was then centrifuged to give a precipitate, PN_{2s} containing subnuclear particles comparable in density to the original cytoplasmic P_2 fraction.

Viral inoculation. The nuclear fraction (PN_2) or the mitochondrial supernatant (S_2) was inoculated into the pronephric cell cultures and absorbed for 1½–2 hrs. After incubation the inoculum was retained but fresh L-15 medium was added and incubated at 25°.

Electron microscopy. Infected cells were subjected to freezing and thawing three times, the cell debris was sedimented at 5090*g* for 20 min and the supernatant was centrifuged at 100,000*g* for 2 hr. The pellet was resuspended in 1 ml of 2% ammonium acetate and applied to carbon-coated grids. These were negatively stained with 2% potassium phosphotungstic acid, pH 6.5.

Infected pronephric cells for ultrathin sections were scraped from the bottle and sedimented at 800 × g. Cell pellets were fixed in 4% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.3), postfixed in 1% phosphate-buffered osmium tetroxide, embedded in Epon and stained with uranyl acetate and lead citrate.

Bioassay of the viruses. The mitochondrial fraction (P_2) and microsomal fraction (P_3) obtained from infected WMPa cells by the same procedure used for tumor cells were injected separately (0.2 μl) into young embryos of *Rana pipiens* (Shumway stage 18). The nuclear fraction and mitochondrial fraction from the original tumor (RT 208) were also injected into embryos for an oncogenicity test. Noninoculated embryos were used as stock controls.

Results. The primary pronephric culture consisted of predominantly epithelial like cells. These cells have been grown in culture for over a year and still maintain an epithelial nature. They tend to yield a maximum cell population of 6.1×10^6 cells per flask when they show characteristic contact inhibition. The population doubling time of the WMPa cells was calculated at 90 hr (Fig. 1). Chromosome counts of the WMPa cells indicated that the cells were truly diploid in early passages and then became aneuploid at later passages having a major mode of 23 chromosomes (Fig. 2).

The tumorigenicity of the noninfected pronephric cells was tested after 15 passages when 2×10^6 cells were resuspended in 1 ml of L-15 medium and injected intraperitoneally into Shumway stage 25 larvae. Cells were also inoculated into the brains of larvae or intraocularly into adult *Rana pipiens*. In all cases the cells were found to be nontumorigenic.

There were no cytopathic effects in the WMPa cells (Fig. 3) inoculated with the nuclear fraction (PN_2) from the tumor but pronephric cells exposed to the mitochon-

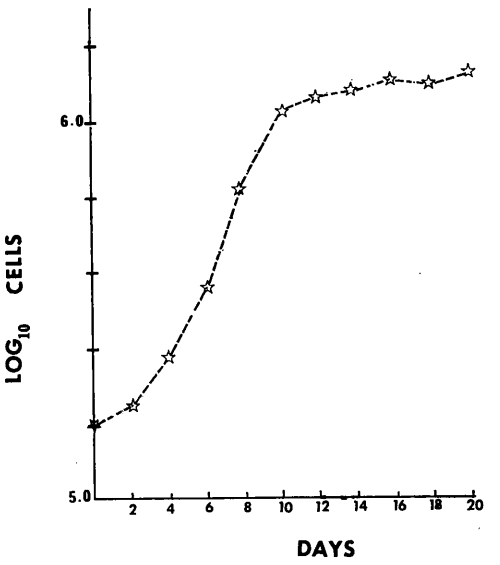

FIG. 1. Growth curve of WMPa cells. Each point represents cell counts of three 74 cm² Falcon flasks. Population doubling time is estimated to be 90 hr.

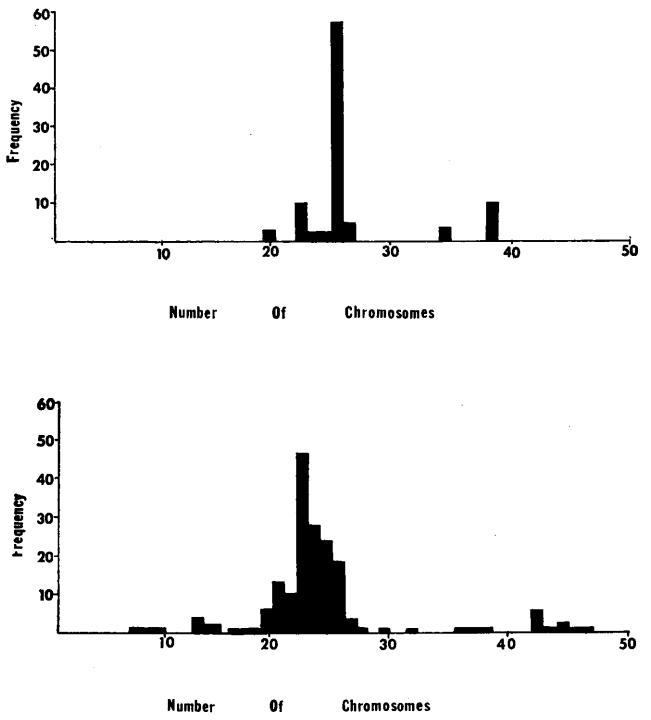

FIG. 2. Chromosome counts for WMPa cells. This cell line showed a true diploid number (26) at the 7th cell passage (upper graph) but at the 15th passage (lower graph) the line showed tendency towards aneuploidy.

drial supernatant (S_2) fraction showed cytopathic effects 12 days after infection. Two types of intranuclear inclusions were observed. About 5% of the infected cells had nuclei with marginated chromatin and eosinophilic nuclear inclusion bodies resembling the type A found in the herpes infected Lucké tumor (Fig. 4). Another type was a type B basophilic intranuclear inclusion surrounded by a halo (Fig. 5). Final CPE produced rounded cells which became pycnotic and detached. The same CPE could be induced in an uninfected pronephric culture by inoculation with frozen and thawed infected cells.

In the first two passages only negatively stained icosahedral viruses (resembling an adeno virus) with an average diameter of 70 nm and solid capsomeres that formed a triangle on a 3 fold axis, were frequently seen in nuclei of infected cells (Fig. 6). In the third and later passages, a herpes virus was also seen together with the adeno-like virus (Fig. 7). The adeno-like virus infection seemed to be largely abortive in these passages as only empty capsids of this virus were found in thin sections. Likewise, the type B inclusions were not observed after the third virus passage. At the same time mature herpes virus particles predominated in the infected cells from later passages and the adeno-like virus was not seen.

Injection of cell fractions from early passages of these two viruses into embryos was lethal. More than 50% of the embryos were killed by the end of 2 weeks when injected with the microsomal fraction of the infected pronephric cells. Those injected with a mitochondrial fraction of the infected cells showed 50% lethality within 5 weeks. The diseased embryos exhibited turgid edema and subdermal hemorrhage. No survivors were found in the group injected with the microsomal fraction after 6 weeks (Table I).

Coincident with the apparent abortion of

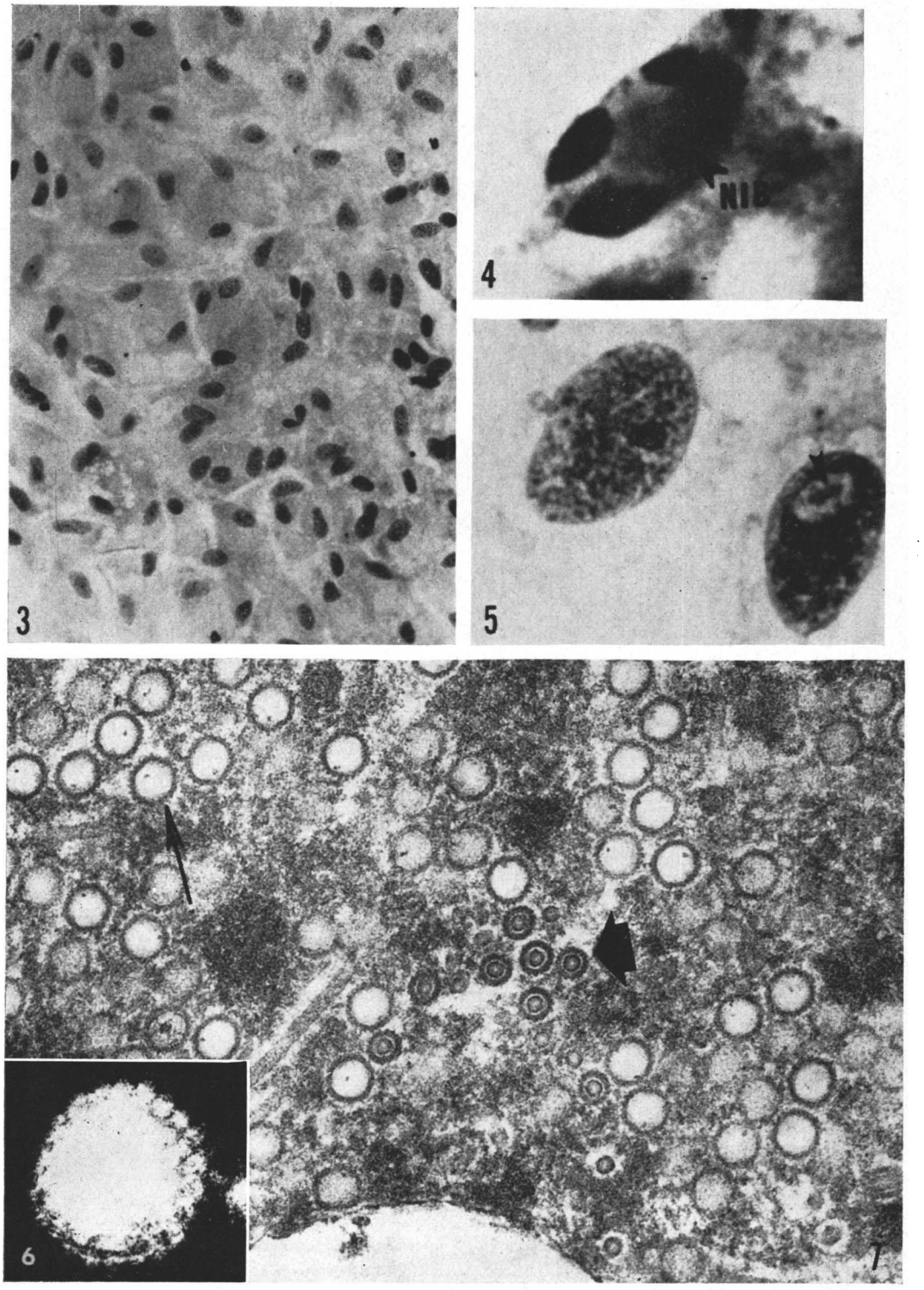

FIG. 3. Noninfected pronephric (WMPa) cells showing epithelial like character plus a few fibroblasts at 12 days after subcultivation. Carnoy fixed, Jenner-Giesma stain (×100).

TABLE I. Inoculation of *Rana pipiens* Embryos with a Mixed Viral Infection of Embryonic Kidney Cells or Tumor Viral Fractions.

Viral source	Number injected	LD_{50} (Weeks)	Survivors (6 weeks)	
Tissue Culture Fractions (1st virus passage)				
Microsomal fraction (P_3)	25	3	0	
Mitochondrial (P_2)	26	5½	10	
Controls	12	—	9	
Tumor Cell Fractions				
Nuclear (PN_2)	40	3	6	
Mitochondrial P_2 10^{-1}	92	4–6	8	
Mitochondrial P_2 10^{-2}	59	3½–4½	6	
Controls	42	—	39	
Tissue Culture Fractions (4th virus passage)				
Mitochondrial P_2 10^{-1}	30	—	28	
Controls	12	—	10	
Tissue Culture Fractions (6th virus passage)				(3 mo)
Mitochondrial P_2 10^{-1}	36	—	36	31
Controls	26	—	25	19

the adeno-like virus and the increase of herpes virus in thin sections of the cell, the lethal effect disappeared. Similar injection of P_2 infected cell fractions from later passages into new stage 18 embryos failed to produce the lethal response. Embryos survived well beyond the 6 week period with no symptoms of the earlier response (Table I).

The lethal effect was presumably obtained from a virus derived from the original tumor fractions and not from the pronephric cell line. Additional embryos were injected with the viral containing nuclear (PN_2) or mitochondrial (P_2) fractions of the original tumor. Injections of fresh, ice stored (1 week) or frozen tumor fractions (1 month) into embryos were ultimately lethal after causing symptoms identical to those described for the infected cultures. In most groups the LD_{50} occurred 3–4½ weeks after injection and all embryos had died just after 6 weeks (Table I).

Discussion. The WMPa cell line is the first established from embryonic pronephric kidney cells. The growth rate resembles that of other amphibian cells. The aneuploid nature of this cell line seems to fit Hayflick's idea of an established cell line (12). However, the aneuploid cells were nontumorigenic upon injection back into the host. The significance of this cell line is its permissiveness to different frog renal tumor viruses.

The adeno-like virus isolated probably represents a 4th virus associated with the Lucké tumor: FV-3, FV-4, the papova-like virus and the present isolate. This virus is judged as an adeno-like virus because of its

FIG. 4. An infected nucleus from WMPa cells showing Type A eosinophilic inclusion body with clumped, marginated chromatin. NIB: nuclear inclusion body (×420).

FIG. 5. A basophilic Type B inclusion body surrounded by a halo found in an infected nucleus (arrow) (×340).

FIG. 6. A negatively stained adeno-virus with solid capsomeres and an eccentric core. Potassium phosphotungstic acid stain. Bar represents 100 nm (×430,000).

FIG. 7. Thin section of an infected cell nucleus showing two types of viral capsids. The double shelled particle (fat arrow) has an average diameter of 70 nm resembling an adeno-virus. The smallest circular particle is identical in size to the 35 nm core of the "adeno-like virus" and was only found in association with the latter. The other 90 nm empty capsids (long arrow) are immature herpes virus (×90,000).

overall diameter of 70 nm with a 35 nm core, icosahedral symmetry, solid capsomeres, and production of basophilic intranuclear inclusions. It is highly cell-associated since most of the viral particles could not be released into the medium unless the infected cells were subjected to freezing and thawing. The rounded cell CPE seen after infection also resembles the CPE of other adeno viruses (13).

This adeno-like virus has a very restricted host cell susceptibility. Attempts to infect other cell lines *Xenopus* kidney (A-6), *Xenopus* liver (A-8) and *Rana* adult kidney (PWK) were unsuccessful judging from the lack of CPE. That the adeno virus infection became abortive in later passages of WMPa may be due to inhibition by increased proliferation of the herpes virus.

The morphology of the herpes virus isolated in later passages resembles the oncogenic herpes virus found in Lucké inclusion tumors. The result of the current bioassay indicated the effect of another virus in the inoculum besides the herpes virus, as the herpes virus alone will not cause this response in embryos (8, 9). The new adeno-like virus that was recovered from the infected pronephric cells most probably came from the original tumor fraction, since the injection of the tumor fraction caused similar pathology in the embryos. A similar pathology was observed in FV-3 injected embryos (14). The presence of FV-3 was ruled out because FV-3 could not be recovered from the current tissue cultures, and the absence of cytoplasmic inclusions in the infected cells.

Another adeno virus, FAV-1, has been isolated by Clark *et al.* (7) from a granulomatous kidney of *Rana pipiens* after passage in the newt. It differs from the present isolate in its host cell susceptibility, slightly different size and its effect on embryos. FAV-1 has a diameter of 80 nm and only replicates in the turtle heart (TH-1) cell line. When FAV-1 was injected into frog embryos there were no apparent pathological effects. Whether FAV-1 and the present isolate are antigenically related remains to be determined.

The rare occurrence of the adeno-like virus in *Rana pipiens* argues against its significance in the etiology of the Lucké tumor. It probably represents a passenger virus in the tumor rather than serving as a helper virus. This investigation also confirms Clark's finding of an adeno virus in poikilothermic animals.

Summary. The first established frog pronephric cell line was exposed to tumor cell fractions of the oncogenic (nuclear inclusion body) phase of the Lucké renal adenocarcinoma. A mixed infection of the herpes virus and adeno-like virus was obtained. When tested by embryonic bioassay, the original tumor fractions and the first passages of the viruses in tissue culture produced subdermal hemorrhage, turgid edema and total deaths in 3–6 weeks. In subsequent passages the adeno-like virus appeared to abort and the herpes virus predominated. Bioassay of these and later passages produced no trauma within 3 months.

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