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Isolation and Characterization of Viruses from the Kidneys of *Rana pipiens* with Renal Adenocarcinoma Before and After Passage in the Red Eft (*Triturus viridescens*)

H. FRED CLARK,[1] JAMES C. BRENNAN, ROBERT F. ZEIGEL, AND DAVID T. KARZON[2]

Departments of Pediatrics, Microbiology, and Pathology, School of Medicine, State University of New York at Buffalo, Buffalo, New York 14214

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Viruses were isolated from kidneys of normal and renal tumor-bearing Vermont *Rana pipiens* after subinoculation into red eft newts (*Triturus viridescens*). Organs of efts inoculated with viable cell suspensions from four of seven tumor-bearing kidneys yielded virus (LT-1, -2, -3, -4) when inoculated into TH-1 (*Terrapene* heart) cell culture. One tumor-bearing kidney also yielded virus (L-4) by direct inoculation into TH-1 cells. An additional isolate (L-5) was obtained from 1 of 52 normal Vermont frog kidneys inoculated directly into TH-1 cells. LT-1 was propagated with cytopathic effect (CPE) in each of 38 cell types tested, of fish, amphibian, reptilian, avian, and mammalian origin, at 23 or 30 C. LT-1 through LT-4, L-4 and L-5, and FV-1 through FV-3 each induced similar CPE in all cells tested. LT-2, however, induced CPE that progressed at a slower rate than that caused by the other isolates and produced smaller plaques (<0.8 mm) under starch gel overlay. Each of the viruses replicated to high titer in embryonated eggs incubated at 30 C. The viruses also grew in efts and adult newts, but not in bullfrog (*Rana catesbeiana*) tadpoles or adult leopard frogs. Tumor induction in adult leopard frogs inoculated with LT-1 was not demonstrated. Electron microscopic observations of LT-1 and LT-2 viruses revealed cytoplasmic particles, hexagonal in cross section, approximately 120 to 140 mμ in diameter, containing a dense nucleoid. LT-1 and LT-2 viruses were indistinguishable from FV-1 and *Tipula* iridescent virus. LT-1 was presumed to be a deoxyribonucleic acid virus on the basis of 5-bromodeoxyuridine inhibition. The isolates were ether-sensitive. On the basis of biological, physicochemical, and antigenic similarities, LT-1 through LT-4, L-4, L-5, FV-1 through FV-3, and isolates recently recovered from the bullfrog and the newt may represent strains of the same amphibian cytoplasmic virus.

A viral etiology was originally suggested for the renal adenocarcinoma of the leopard frog, *Rana pipiens*, on the basis of intranuclear inclusions and apparently successful tumor transfer with cell-free filtrates (12, 13). In recent years, several viral isolates obtained in the course of studies of this tumor have been described. Viruses designated FV-1 to FV-3 and FV-9 to FV-19 have been isolated from tumor homogenates, normal liver homogenates, and spontaneously degenerating cell cultures of normal kidneys of *R. pipiens* (8, 9). These agents are characterized by growth at 23 to 30 C in the cytoplasm of cells of numerous cell culture types. Viruses designated FV-4 to FV-8, which characteristically grow at a very slow rate and only in cell cultures derived from embryonic anurans, have been isolated from tumor homogenates and urine pools of frogs bearing tumors with intranuclear inclusions. These viruses appear to replicate in the nuclei of infected cells (17).

In 1952, Rose and Rose reported the production of rapidly growing anaplastic tumors after im-

[1] Present address: Virology Laboratory, Children's Hospital of Buffalo, Buffalo, N.Y. 14222.

[2] Recipient of Research Career Award AI-1136 from the National Institute of Allergy and Infectious Diseases.

plantation of frog renal adenocarcinoma into the newt *Triturus viridescens* by a variety of routes (19). A particularly high percentage of tumor graft "takes" was obtained in the eft, the immature terrestrial stage of the newt. The present study was initiated on the premise that if a virus were indeed involved in the etiology of the Lucké tumor, the rapidly growing anaplastic type of newt tumor might provide a more promising source of virus than the rather slow-growing frog kidney tumor. In an attempt to test this hypothesis, cell suspensions from leopard frog kidneys with tumors or precancerous changes were inoculated into red efts. Attempts were subsequently made to isolate virus from eft organ suspensions by inoculation into the turtle cell line TH-1 (1). In parallel studies, attempts were made to isolate virus by direct inoculation of the frog kidney suspensions into TH-1 cell cultures and by preparations of cell cultures directly from the frog kidneys. Six viruses were recovered in the course of these studies. Their isolation and preliminary characterization are reported. We also compared certain properties of the viruses isolated in our laboratory with those of frog viruses FV-1 through FV-3, isolated from frog kidneys by other methods (9) and kindly supplied to us by Allan Granoff and Keen Rafferty.

MATERIALS AND METHODS

Frogs. "Vermont" frogs were adult *R. pipiens* purchased from J. M. Hazen, Alburg, Vt. "Wisconsin" frogs were purchased from the Lemberger Co., Oshkosh, Wis. Small numbers of *R. pipiens* from western New York were collected at Chautauqua Lake (Chautauqua County) and Williamsville (Erie County), N.Y. Frogs were sacrificed immediately upon receipt or occasionally after holding for as long as 6 months. All amphibians were kept at 23 C ± 2 C. Frogs held for extended periods were fed crickets at weekly intervals. Bullfrog tadpoles (approximately 10 cm, hind legs only protruded) were purchased from the General Biological Supply Co., Chicago, Ill.

Newts. In early experiments, red efts of the newt subspecies *T. viridescens dorsalis* (indigenous to North and South Carolina) were purchased from the Lemberger Co., Oshkosh, Wis. In later studies, red efts and adult newts of the subspecies *T. viridescens viridescens* were purchased from the General Biological Supply House, Inc., Chicago, Ill. Newts were not fed during the course of experiments.

Cell cultures. The *Terrapene* heart cell line, TH-1, was developed in this laboratory and is described elsewhere (1). Rainbow trout gonad cells were purchased from the American Type Culture Collection and maintained at 23 C. The origin of other cell cultures is listed in Table 2.

Frog kidney studies. Frogs were sacrificed by pithing or by anesthetizing with Chloretone or MS 222 (Sandoz). MS 222 was found to alter the histological appearance of the kidney by causing severe edema. Pithing was the preferred method of sacrifice.

The kidneys were examined under a dissecting microscope, and a portion of those with suspected tumors was fixed for histological study. The remaining tissue of the suspected tumor-bearing kidneys was triturated in amphibian Ringer's solution or dispersed in trypsin. Trypsinized cells (yield 2.0 to 20.0 × 10^6 cells per kidney) were suspended in Eagle's basal medium with 10% fetal calf serum (BME-FCS10) or the frog cell growth medium of Rafferty (*personal communication*).

Cells were plated on cover slips in Leighton tubes for studies of direct outgrowth and inoculated into TH-1 or Rainbow trout gonad cell cultures and into efts.

Nucleic acid inhibition studies. 5-Bromodeoxyuridine (BUDR) was purchased from Calbiochem, Los Angeles, Calif. Thymidine was purchased from the Nutritional Biochemicals Corp., Cleveland, Ohio. Herpes simplex virus (HSV), strain HF (deoxyribonucleic acid control virus), and vesicular stomatitis virus (VSV) strain Indiana (ribonucleic acid control virus) were propagated in primary chick embryo cell cultures.

RESULTS

Tumor incidence. A total of 175 adult Vermont *R. pipiens* were sacrificed and examined for kidney tumors. Thirteen tumors were detected grossly and one tumor was detected histologically. The tumor incidence was higher in female (12 of 97, 12.4%) than in male frogs (2 of 78, 2.6%).

Observations on cell cultures derived from tumor-bearing and normal frog kidneys. It was suggested that frog kidney cells are especially likely to yield virus when cultivated in vitro (8). In this study, direct cell culture studies were performed on trypsinized cell suspensions from the kidneys of 10 frogs with histologically confirmed tumors and of 9 normal Vermont frogs. Three of the normal frogs yielded little or no cell growth. Two kidneys with tumors also yielded very limited cell growth (F82 and F129); both yielded virus (Table 1). However, the virus-yielding tumors F119 and F209 (Table 1) grew in cell culture. There was little difference in the appearance of cell cultures derived from tumor-bearing and normal kidneys. In both, a mixed population of small epithelial cells and large flattened epithelial cells, often containing several nuclei, was observed. Enlarged nucleoli, frequently surrounded by a clear halo-like area, were observed in from 1 to 50% of cells in cultures of both tumor and normal tissue. These nucleoli were especially common in the multinucleated cells. Their incidence was not correlated with the presence of neoplasia. Multinucleated giant cells and altered nucleoli have previously been described primarily in cultures of frog renal

TABLE 1. *Inoculation of efts (Triturus viridescens) and TH-1 (Terrapene heart) cells with Rana pipiens kidney cells*[a]

Frog no.	Gross and histological examination of kidney	Virus isolation in TH-1 cells	No. of efts inoculated	Eft disposition	Eft organs in TH-1 cell cultures[b]
82	5 mm tumor, adenocarcinoma	−	4, pyribenzamine[c]	3 dead, days 1, 19, 19 1 sacrificed, day 16	ND[d] L, K, S, + LT-1 virus
96	5 mm tumor, adenocarcinoma	−	12, pyribenzamine	4 sacrificed, day 21 8 dead, days 18–25	L, H− ND
97	Small (approx. 1 mm) tumor nodules, no histological examination	−	6, pyribenzamine	2 sacrificed, day 21 4 dead, day 18	L, H− ND
119	Small (≤2 mm) tumor nodules, early proliferative changes	−	7, pyribenzamine	2 dead, day 8 5 dead, days 3–10	L, K, S, T, + LT-3 virus ND
129	6 mm tumor and smaller nodules, adenocarcinoma	−	8, pyribenzamine	1 dead, day 9; 1 dead, day 10 6 dead, days 3–13	L, Lu, H, S, K, O, + LT-2 virus ND
203	Approx. 2 mm tumor nodules, adenocarcinoma	−	2, pyribenzamine 2, water	2 sacrificed, day 15 1 dead, day 10; 1 sacrificed, day 15	L, H, K, − L, H, K, −
209	L kidney, small (<1 mm) nodules, early proliferative changes	+ L-4a virus	2, pyribenzamine 2, water	1 dead, day 12 1 dead, day 12 1 dead, day 16; 1 sacrificed, day 16	L, H, K, − L, H, K, + LT-4a virus L, H, K, −
209	R kidney, small (<1 mm) nodules, early proliferative changes	+ L-4b virus	2, pyribenzamine 2, water	1 dead, day 14 1 sacrificed, day 16 1 dead, day 16; 1 sacrificed, day 16	L, H, K, − L, H, K, + LT-4b virus L, H, K, −
5 separate normal kidneys, histologically normal		−	4–6 per kidney in pyribenzamine or water	All sacrificed or dead, days 15–24	2–4 efts inoculated with each kidney L, H, K, −

[a] TH-1 cell cultures were inoculated with 1.2 to 16.0 × 10^4 cells. The eft inoculum was one-half the cell culture inoculum.
[b] L = liver, H = heart, K = kidney, S = spleen, T = testis, Lu = lung, O = oviduct.
[c] Held in distilled water containing 0.05 mg of pyribenzamine per 100 ml.
[d] ND = not done.

tumor cells (4, 7, 16). It is possible that our cultures of "normal" kidneys may have contained foci of tumor cells not detected by histological examination. Conversely, it is also likely that kidney tumors dispersed for cell culture contained portions of normal kidney tissue. Spontaneous degeneration suggestive of viral cytopathic effect was not observed.

Isolation of virus from tumors and normal kidney cell suspensions inoculated into efts and TH-1 cells. Suspensions of viable kidney cells from seven frogs with tumors and five normal frogs were inoculated into red efts and into TH-1 cell cultures. Tissues of efts dying or sacrificed after 15 or 16 days were subinoculated into TH-1 cell cultures (Table 1).

Cytopathic agents were isolated from the organs of efts inoculated with four of the seven tumor cell suspensions. These agents were designated "LT" for *Lucké-Triturus*, and numbered 1 through 4. No cytopathic agents were isolated from efts inoculated with cell suspensions from five normal kidneys. In one instance, both kidneys from a single frog (no. 209) yielded virus (L-4) by direct inoculation of tumor cells into TH-1 cell culture. Despite this, only two of eight efts were infected (LT-4) when inoculated with this tumor cell suspension. Infected efts commonly yielded virus from all organs tested. Virus was not isolated from a total of 42 uninoculated control efts sacrificed in the course of 13 different experiments.

Of the four cancerous kidneys yielding virus directly or indirectly, two had adenocarcinomas and two had early neoplastic proliferative changes with occasional tubular cells showing vacuolated cytoplasm. The latter change was previously described as "clear cell pathology" by Duryee (5). Duryee suggested that such "clear cell pathology" might be caused by "passenger virus" infection, but he presented no supporting evidence.

Severe subcutaneous hemorrhages noted in several infected efts were first considered to be specific virus lesions. However, similar hemorrhages were subsequently noted in uninoculated control efts dead from unknown causes. Attempts to recover virus from such control efts were uniformly negative.

Virus-yielding efts often died between 1 and 15 days after inoculation, whereas no control efts inoculated with normal kidney cells died prior to the 15th day. No macroscopic evidence of growth of tumor was observed in efts (histological studies were not performed). Of the efts inoculated with each frog cell suspension, all or one-half were kept in water containing 0.05 mg of pyribenzamine per 100 ml, a concentration reported to inhibit the inflammatory response and to permit frog tumor transplant "takes" in newts (19). The effect of pyribenzamine on virus recovery was uncertain. However, two of four efts in pyribenzamine inoculated with cells of tumor F209 yielded virus, whereas no virus was recovered from four efts kept in water and inoculated with the same cell suspension.

Attempts to isolate virus from tumor and normal kidney suspensions inoculated into TH-1 cells only. Kidney suspensions of 6 tumor-bearing and 52 normal Vermont frogs, 32 normal frogs from Western New York, and 4 normal frogs from Wisconsin were inoculated into TH-1 cell culture only. Liver suspensions from 18 New York frogs, and lung and spleen suspensions from 5 New York frogs, were also inoculated into TH-1 cells. The kidneys considered to be normal had been examined histologically. One of 52 normal Vermont frog kidney suspensions yielded a cytopathic agent (L-5) but none of 6 tumorous kidney suspensions was positive. No agents were isolated from non-Vermont frog tissues.

Propagation of viruses in cell culture. LT-1 through 4 and L-4 and 5 viruses were successfully propagated with cytopathic effect in every cell line tested (total of 38 cell types tested) when incubated at 23 or 30 C. Representative fish, amphibian, reptilian, avian, and mammalian cell cultures were included. LT-1 virus has been most thoroughly studied. The cell types in which LT-1 has been propagated are listed in Table 2 (2, 10, 17, 21). The cytopathic effect (CPE), essentially similar in every cell type, consisted of rounding up of cells which became pycnotic and separated from each other, leaving a network of fine cytoplasmic processes. Staining with hematoxylin and eosin revealed basophilic cytoplasmic inclusions in many cells. Inclusions varying widely in size, shape, and number often appeared before other cytopathic changes. Nuclear inclusions were rarely seen, although marginated chromatin was often observed and nuclei became very pycnotic in the terminal stages. A slightly different form of CPE was observed in the fathead-minnow cell line only. In this cell line, CPE consisted of small "punched out" holes in the cell sheet. Rounded cells with cytoplasmic inclusions were observed at the margins of these "punched out" areas. The CPE caused by LT-1 through 4, L-4 and 5, and FV-1 through 3 was compared in H + E stained cover-slip preparations of 13 cell lines of poikilothermic vertebrate origin and in primary chick embryo and rhesus monkey kidney cell cultures. No differences in CPE caused by the different isolates were observed in any of these cell systems. However, lesions induced by LT-2 virus invariably progressed at a slower rate than those induced by

TABLE 2. *Cell culture systems susceptible to LT-1 virus as indicated by cytopathic effect*[a]

	Cell system and species of origin	Normal cell propagation temperature	No. of virus passages[b]
		C	
Fish	Rainbow trout gonad, *Salmo gairdneri* (21)	23	1
	Fathead minnow, *Pimephales promelas* (10)	23	1[c]
	Bluegill fry, *Lepomis microchirus* (Wolf, *unpublished*)	23	1
	Grunt fin, *Haemulon flavolineatum* (2)	23	1
Amphibian	Bullfrog tongue (FT), *Rana catesbeiana* (22)	23	1
	Pickerel frog kidney (KERS), *R. palustris* (17)	23	1
	Lucké carcinoma, *R. pipiens* (17)	23	1
Reptilian	Box turtle heart (TH-1A), *Terrapene carolina*[d]	23	12[c]
	Box turtle heart 1 (TH-1B_1)	23	1
	Box turtle heart 1 (TH-1W)	30	1
	Box turtle heart 4 (TH-4)	23	1[c]
	Box turtle heart 4 (TH-4W)	30	1[c]
	Box turtle lung 4 (TL-4)	23	1[c]
	Box turtle spleen 4 (TS-4W)	30	1
	Box turtle heart 5 (TH-5W)	30	1
	Box turtle kidney (TKW)	30	1[c]
	Grecian tortoise spleen, *Testudo graeca*	30	1[c]
	Side-necked turtle heart 1, *Podocnemius unifilis*	30	1[c]
	Side-necked turtle heart 2	30	1
	Gecko heart, *Gekko gecko*	30	1[c]
	Gecko lung	30	1[c]
	Iguana heart, *Iguana iguana*	36	1
	Iguana liver	30	1
	Primary box turtle kidney	23	1
	Primary cayman kidney, *Caiman crocodilus*	30	2
	Primary iguana kidney	30	1
	Primary amphisbaenian liver, *Blanus cinereus*	30	1
	Primary python kidney, *Python reticulata*	30	1
Avian	Chick embryo fibroblasts, *Gallus domesticus*	36	2
Mammal	Human fibroblast (WI-38)	36	1
	Human amnion (WISH)	36	2
	Human carcinoma (KB)	36	5
	Human amnion (AV_3)	36	3
	Human sternal marrow (Detroit 6)	36	1
	Green monkey, *Cercopithecus aethiops*	36	5
	Baby hamster kidney (BHK), *Mesocricetus auratus*	36	1
	Primary rhesus monkey kidney, *Macaca mulatta*	36	4[c]
	Primary rabbit kidney, *Oryctolagus caniculus*	36	4

[a] LT-1 virus-inoculated reptile, amphibian, and fish cell cultures were incubated at 23 C; mammalian and avian cell cultures at 30 C. Cultures listed are continuous lines except where noted.

[b] All passages listed supported LT-1 virus-induced CPE.

[c] Indicates cell culture system in which H + E stained cover-slip preparations of cells infected with LT-1 through 4, L-4, and 5, and FV-1 through 3 were compared.

[d] All reptilian cell lines and primary cultures of all species originated in this laboratory.

the other viruses. In some cell systems, LT-2 virus never produced confluent CPE. Typical examples of the CPE induced by LT-1 virus in several host systems are shown in Fig. 1.

Quantitative differences in the susceptibility of different cell types were suggested by differences in the rate of appearance of lesions. The time required for cytopathic involvement of the entire cell sheet in cultures inoculated with approximately $10^{5.0}$ $TCID_{50}$ of LT-1 virus varied from 1 to 2 days in most reptilian cell lines and from 3 to 7 days in most mammalian, avian, amphibian, and fish cells. Because virus stocks were maintained in TH-1 cells, the apparently greater

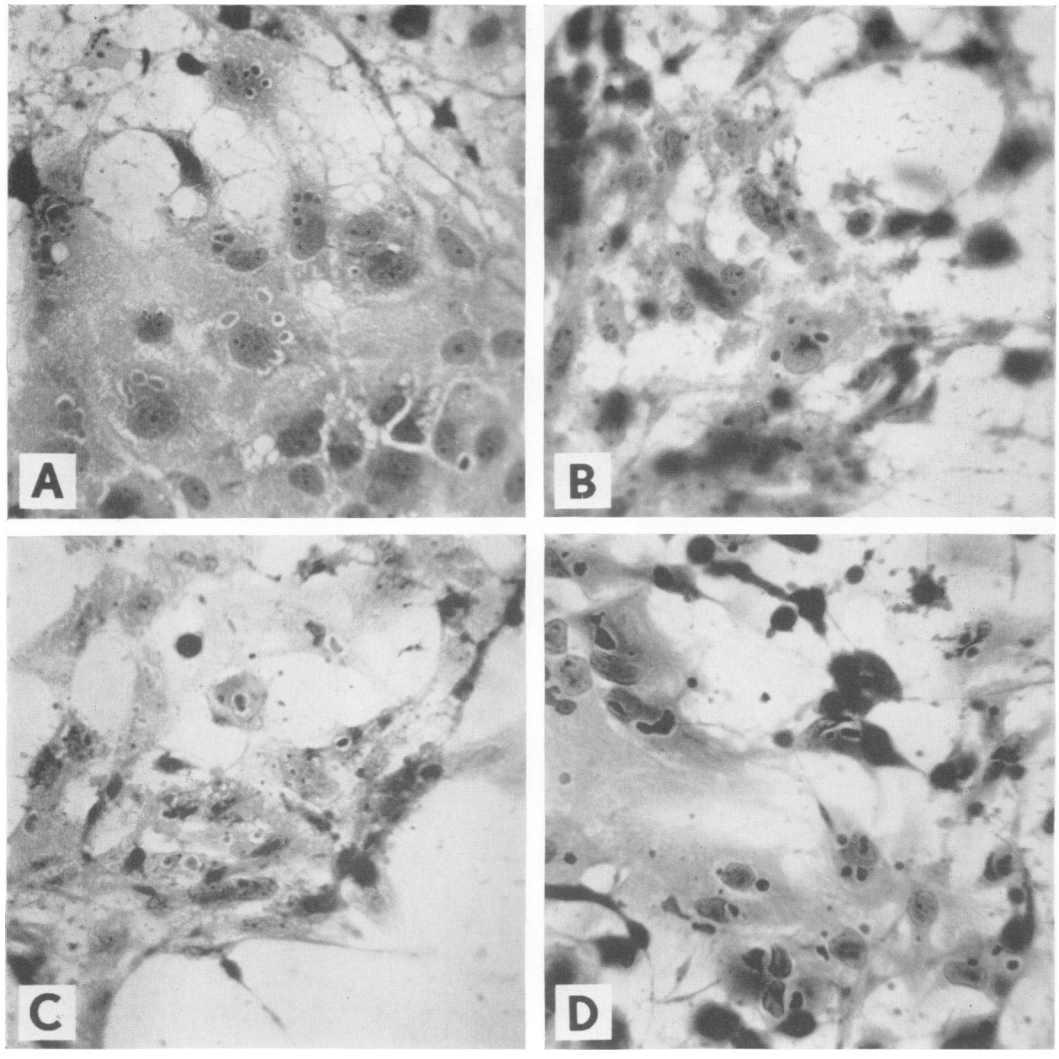

FIG. 1. *Cytopathic effect of LT-1 virus. (A) Terrapene heart (TH-1) cells at 23 C. Cell destruction and paranuclear cytoplasmic inclusions. Indistinct cell margins are normal. (B) Gecko lung (GL-1) cells at 23 C. Paranuclear or scattered cytoplasmic inclusions and rounded pycnotic cells. (C) Primary chick embryo cell culture at 30 C. Cell destruction with scattered cytoplasmic inclusions of widely varying size. (D) Primary rhesus monkey kidney cell culture at 30 C. Large paranuclear cytoplasmic inclusions, nuclei with marginated chromatin, and dense pycnotic rounded cells.*

susceptibility of reptilian cells may reflect adaptation to this turtle cell line.

In addition to causing an unusually slow-developing CPE, LT-2 also differed from the other isolates in producing unusually small plaques in certain cell lines. LT-2 produced plaques less than 0.8 mm in diameter after 9 days of incubation under starch gel overlay at 23 C in the turtle cell lines TH-1A (11), TH-$1B_2$, and in BHK cells. LT-2 produced normal-size plaques, 0.8 to 2.0 mm in diameter, in the turtle cell lines TH-1W, TH-5W, and TGS, in the fish cell line FHM, and in primary chick cell culture. LT-2 was isolated from the kidneys of eft no. 79 inoculated with tumor F129. Plaque assays of virus from the organs of this eft and eft no. 76 inoculated with the same tumor were performed after 24 months of storage at −40 C. Virus was not reisolated from the kidneys of eft 79, but virus was reisolated from the liver of this eft and from the liver, kidneys, and oviducts of eft 76. In each case, the virus population produced predomi-

nantly large plaques. It would appear that LT-2 stock virus represents a chance isolation of a small-plaque variant either directly from the eft kidney or in the course of passage of the virus in TH-1A cell culture prior to the initial plaque assay at the fourth passage level.

Growth of virus in embryonated eggs. Preliminary studies were performed with eggs incubated at 36 C. LT-1 inoculated into the allantoic sac of 7-day embryonated eggs caused no deaths during 12 days of observation. LT-1 inoculated onto the chorioallantoic membranes (CAM) of 7-day embryonated eggs caused no visible lesions detectable 6 days after inoculation.

Subsequently, 7-day embryonated eggs were inoculated via the yolk sac with approximately $10^{6.0}$ $TCID_{50}$ of viruses LT-1 through 4, L-4, and FV-1 through 3, and were incubated at 30 C. Most embryos died on the 4th or 5th day, except those inoculated with LT-2, which died between the 5th and 7th days. Uninoculated control eggs held at 30 C began to die on the 6th day, presumably because of the unfavorable incubation temperature. Tissues from chick embryos dead 5 days after inoculation with LT-1 were harvested and titrated in TH-1 cell culture. Virus yields ($TCID_{50}$/g or ml) were as follows: yolk sacs, $10^{9.5}$; CAM, $10^{9.5}$; chick embryos, $10^{6.2}$; and allantoic fluid, $10^{6.5}$. The yields from CAM and yolk sac were at least 100-fold greater than the maximal titers obtained in TH-1 cell culture. No specific lesions were seen on the CAM of these eggs or of eggs inoculated into "dropped" CAM and incubated at 30 C.

Growth of viruses in poikilothermic animals. Several of the amphibian viruses were inoculated into red efts and mature newts, Vermont and Wisconsin *R. pipiens* and bullfrog (*R. catesbeiana*) tadpoles in short-term experiments. Some representative titers found in livers, kidneys, and spleens are shown in Table 3.

LT-1 through 4, L-4, and FV-1 through 3 grew to high titer in red eft newts. At death, efts occasionally showed subcutaneous hemorrhage or enlarged spleens, or both. The specificity of these lesions and of the early deaths observed in some virus-inoculated animals is uncertain.

Adult newts responded in a more irregular manner to virus inoculation. Virus grew to high titer in each organ tested in many animals, but other individuals were apparently completely refractory to infection. Virus was recovered from one uninoculated control newt. The uninoculated animals were held in a separate room from that containing inoculated animals, but laboratory contamination cannot be ruled out with certainty.

TABLE 3. *Growth of amphibian viruses in amphibians*[a]

Virus	Red efts				Adult newts				Vermont *Rana pipiens*			
	Disposition of animal[b]	Recovery of virus[c]			Disposition of animal	Recovery of virus			Disposition of animal	Recovery of virus		
		L	K	S		L	K	S		L	K	S
LT-1	d 9	6.0	5.0	5.0	s 21	6.0	5.0	5.0	s 21	<0.5	<0.5	<0.5
	d 11	5.5	5.0	4.0	s 21	<0.5	<0.5	>1.0	s 21	<0.5	<0.5	<0.5
LT-2	d 9	6.5	6.0	4.0	d 20	ND[e]	ND	ND	s 21	<0.5	4.5	<0.5
	d 10	5.0	4.5	2.0	s 21	<0.5	<0.5	<0.5	s 21	<0.5	<0.5	<0.5
L-4	d 12	1.0	3.5	1.0	s 21	>6.5	3.0	4.0	d 2	ND	ND	ND
	s 21	4.0	4.0	3.0	s 21	<0.5	<0.5	<0.5	s 21	<0.5	3.5	<0.5
FV-1	s 21	3.5	3.5	4.5	s 21	5.0	3.0	3.0	s 21	<0.5	<0.3	<0.5
	s 21	5.0	5.5	5.5	s 21	>1.0[d]	>1.0	>1.0	s 21	<0.5	<0.5	<0.5
BMECS[f]					s 21	<0.5	<0.5	<0.5	d 2	ND	ND	ND
					s 21	<0.5	<0.5	<0.5	s 21	<0.5	<0.8	<0.5
Uninoculated	d 17	<0.5	<0.5	<0.5	s 21	<0.5	<0.5	<0.5	s 21	<0.5	<0.5	<0.5
	d 17	<0.5	<0.5	ND	s 21	<0.5	<0.5	<0.5	s 21	<0.5	<0.5	<0.5
	d 45	ND	ND	ND	s 21	<0.5	2.5	3.5				

[a] All animals inoculated with $10^{5.0}$ to $10^{6.0}$ $TCID_{50}$ of virus stock prepared in TH-1 cells. Dose divided subcutaneously (0.01 ml) and intraperitoneally (IP), (0.04 ml) in red efts. Other animals inoculated by IP route only (0.10 ml).

[b] Day of death (d) or sacrifice (s).

[c] Titer of virus recovered expressed as $TCID_{50}$ for entire organ titrated in TH-1 cells. L = liver, K = kidney, S = spleen.

[d] Virus recovered from organ suspension but not titrated.

[e] ND = not done.

[f] Cell culture medium (Eagle's basal medium with 10% calf serum) inoculated into control animal.

At 3 weeks after inoculation, virus was recovered in low titer from the kidneys of two of seven virus-inoculated Vermont *R. pipiens*, but from no livers or spleens. Virus was not recovered from liver, spleen, or kidneys of Wisconsin *R. pipiens* (four inoculated with LT-1, LT-2, or L-4 virus) or *R. catesbeiana* tadpoles (16 tadpoles inoculated with LT-1 through 4, L-4, or FV-1 through 3 viruses) 3 weeks or more after inoculation. In no case did virus inoculation appear to cause deaths in adult *R. pipiens* or in *R. catesbeiana* tadpoles.

Fifty Vermont *R. pipiens* were inoculated intraperitoneally with $10^{5.8}$ $TCID_{50}$ of LT-1 (grown in TH-1 cells) and held for 6 to 8 months at 20 to 25 C to determine whether inoculation with virus might have an effect on tumor incidence. A control group of 50 frogs was inoculated with frozen and thawed TH-1 cells only. At the time of sacrifice, only 1 of 27 virus-inoculated frogs had tumorous change in the kidney; 5 of 16 control frogs had gross or microscopic kidney tumors (Table 4), including 1 kidney with tumor and "ballooned" epithelial changes (previously described in tumors F129 and F209). It appeared extremely unlikely that LT-1 virus inoculated into adult *R. pipiens* by the IP route enhanced tumor incidence within the observed incubation period.

Kidney suspensions from all of the frogs listed in Table 4 were inoculated into TH-1 cells with negative results. The one gross tumor and one of the microscopic tumors observed in TH-1 inoculated frogs also yielded no virus after subinoculation into efts.

Attempts to grow virus in mice. Two litters of newborn CFW mice (<20 hr old) were inoculated intracerebrally with $10^{3.5}$ $TCID_{50}$ of LT-1 in 0.01 ml. No virus was recovered in TH-1 cell culture from brain or liver-spleen suspensions prepared from two mice of each litter sacrificed at age 9 days. The remaining mice in each litter (14 mice) remained normal during 30 days of observation.

Adult (30 days old) and newborn mice were inoculated in the hind footpad with LT-1, LT-2, L-4, and FV-1 viruses in doses of approximately $10^{3.5}$ to $10^{5.0}$ $TCID_{50}$. The footpad of the adult mouse has a temperature of approximately 30 C (20). A transient swelling was noted in the feet of most virus-inoculated suckling mice, but all adult mice remained normal. Assay of virus in the inoculated foot, the contralateral foot, the liver, spleen, and blood of suckling mice sacrificed 3, 7, 11, 18, and 24 days after footpad inoculation with LT-1 virus revealed no evidence of viral replication.

The following physiochemical properties were studied.

Morphology. Electron photomicrographic observations were performed on thin sections of pellets of LT-1, LT-2, and FV-1 viruses propagated in TH-1A cells or in CAM of embryonated eggs. Virus particles consisted of dense nucleoids approximately 95 mμ in diameter surrounded by a hexagonal outer structure 120 to 140 mμ in diameter. Particles in various stages of assembly were observed in cytoplasmic synthetic areas (Fig. 2A). Mature particles were observed passing through the cell membrane (Fig. 2B, 2C), acquiring an envelope in the process. Enveloped particles had an outer diameter of approximately 210 mμ. LT-1, LT-2, and FV-1 viruses were indistinguishable from each other and from preparations of *Tipula* iridescent virus examined concurrently.

TABLE 4. *Tumor incidence in Vermont Rana pipiens sacrificed 6 to 8 months after inoculation with LT-1 virus*[a]

No. of frogs	Days after inoculation	Gross tumors	Microscopic neoplastic change
LT-1 virus-inoculated frogs			
22	183	0	0
5	246	0	1
TH-1 cell-inoculated control frogs			
16	246	1	4

[a] LT-1 virus-inoculated frogs received $10^{5.8}$ $TCID_{50}$ (undiluted virus stock grown in TH-1 cell culture). TH-1 cell-inoculated frogs received undiluted (frozen and thawed) cell culture.

Nucleic acid type. Evidence of the nucleic acid type of LT-1 was obtained by studies of inhibition of virus plaques with BUDR incorporated in a starch gel overlay (Table 5). In both TH-1 and chick embryo tissue culture cells, the plaque titer of LT-1 virus was depressed more than 10-fold in the presence of BUDR. HSV titers were equally depressed; VSV titers were not affected by BUDR. In separate experiments (H. F. Clark and C. Gray, *in preparation*), thymidine reversal of BUDR inhibition of LT-1 and HSV replication was also demonstrated. These data suggest that the nucleic acid of LT-1 is DNA.

Heat sensitivity. LT-1 through 3 and L-4 and 5 preparations with titers of $10^{6.0}$ $TCID_{50}$/0.1 ml suspended in BME FCS10 were completely inactivated when exposed to 56 C for 30 min. The effect of incubation of LT-1 (in 50% bovine albumin in Hank's BSS with 2% calf serum) at several lower temperatures has been reported elsewhere (H. F. Clark and D. T. Karzon, Arch. Ges. Virusforsch., *in press*). LT-1 virus titers were stable for at least 5 days at 30 and 23 C, but the

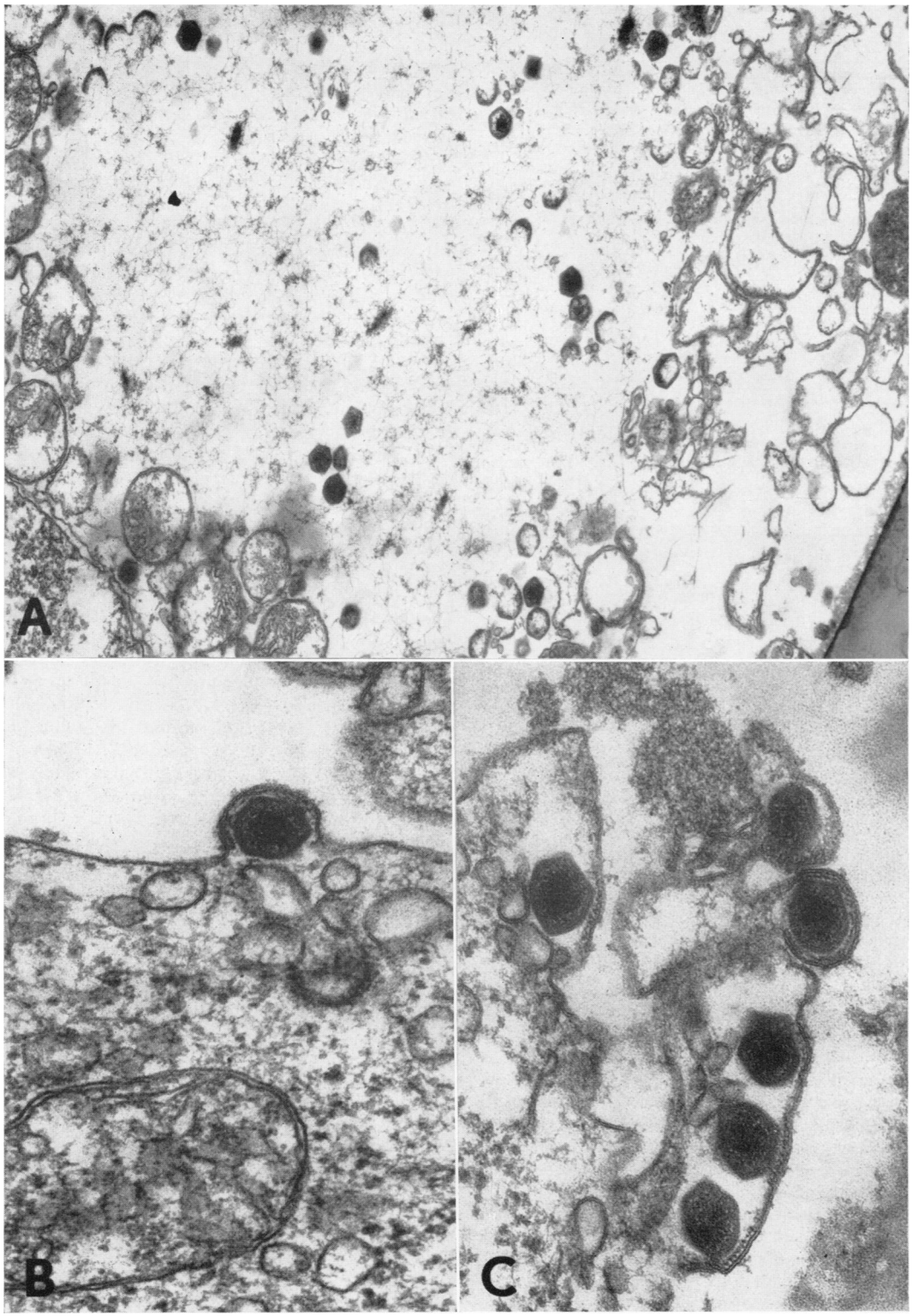

FIG. 2. *Electron micrographs of LT-1 virus in cells of turtle cell line TH-1A. (A) Virus in various stages of maturation in a viroplasmic region within the cytoplasm. Thin filaments (presumably DNA) may be observed to be incorporated into the central regions of the particles. Cytoplasmic organelles apparently do not penetrate the viral synthesis region. Approximately 30,000 × magnification. (B) A mature hexagonal virion is observed passing through the peripheral cell membrane. The internal structure of the virion is resolved as well as the unit membrane structure of the membrane coat. Approximately 75,000 × magnification. (C) Several particles of virus in the process of passing out of the cell. Approximately 75,000 × magnification.*

TABLE 5. *Effect of BUDR (20 μg/ml) in starch overlay on the plaque titer of vesicular stomatitis virus (VSV), herpes simplex virus (HSV), and LT-1 virus grown at 30 C*

Host cells	Virus	Control overlay (PFU/ml)	BUDR overlay (PFU/ml)
TH-1	VSV	1.5×10^7	1.5×10^7
	HSV	3.0×10^4	$<1.0 \times 10^2$
	LT-1	4.6×10^5	$<1.0 \times 10^3$
Chick embryo	VSV	7.2×10^8	7.2×10^8
	HSV	2.1×10^7	$<1.0 \times 10^6$
	LT-1	1.2×10^6	$<1.0 \times 10^5$

virus was totally inactivated in less than 5 days after exposure to 36 C.

Ether sensitivity. Each of the LT and L viruses was exposed to 20% ethyl ether overnight at 4 C. The titers of the preparations of each virus were reduced by $10^{2.5}$ or more, indicating ether sensitivity.

Antigenic studies. Antigenic comparisons of the LT and L viruses and FV-1 through 3 will be reported in detail elsewhere. Extensive reciprocal antigenic cross reactions among all of these viruses have been shown in neutralization (D. E. Lehane, Jr., H. F. Clark, and D. T. Karzon, Virology, *in press*), complement-fixation, and gel diffusion systems (S. Kaminski, Thesis, State University of New York at Buffalo, 1968).

DISCUSSION

Six viruses were isolated during the course of studies of kidney tumor-bearing and normal Vermont *R. pipiens*. These viruses are similar in morphology, nucleic acid type, heat and ether lability, cell culture host range, temperature optimum, cytoplasmic site of replication, and antigenic composition to FV-1 and FV-2 viruses, isolated from kidney cell cultures of "normal" *R. pipiens* from Wisconsin (3, 8), and also to FV-3 virus isolated from a tumor homogenate from a Wisconsin leopard frog (9). Thus, all of the above agents appear to be members of a relatively homogenous group with a single serotype and may be conveniently referred to as the amphibian cytoplasmic virus group until further information permits a more definitive nomenclature. The LT, L, and FV viruses do not resemble the herpes-like viruses observed in nuclear inclusions of renal adenocarcinomas by electron microscopy (6, 15), which were isolated by density-gradient procedures (14) and reported to replicate in frog embryo cell cultures (17).

It has been reported that viruses of the FV-1 type were not readily isolated directly from frog kidney homogenates but were frequently "unmasked" from a presumably latent state by culturing kidney cells in vitro (8). In the present study, while virus was isolated from 1 of 7 tumor-bearing kidneys and 1 of 52 normal Vermont frog kidneys by direct inoculation of tissue homogenates into TH-1 cell culture, three viruses were isolated only after inoculation of red efts with tumor homogenates. It is possible that inoculation of tumor cells into efts is also a means of unmasking latent virus infections of the introduced cells.

The possibility must also be considered that the red efts had overt or latent virus infection prior to the inoculation of frog tissue homogenates. Virus was not isolated from control efts, uninoculated or inoculated with normal frog kidney, but virus was isolated from an uninoculated adult newt. Resistance to virus infection encountered in some adult newts suggested that some of these animals may have acquired immunity from natural infection. Recently, viruses have been isolated in this laboratory directly from several adult newts supplied by one dealer. While these isolates are incompletely characterized, they cause CPE in several host-cell systems incubated at 23 C that is indistinguishable from that caused by LT-1 and FV-1 type agents. Furthermore, antibodies reacting with LT-1 virus in the gel precipitin test have been found in uninoculated adult bullfrogs (S. Kaminski, Thesis, State University of New York at Buffalo, 1968), and viruses similar in several properties to LT-1 and FV-1 have been isolated from bullfrog tadpoles and adults (K. Wolf, *in preparation*). An antigenic relationship of one bullfrog isolate (Wolf) to LT-1 and FV-1 has been demonstrated by agar gel immunodiffusion (P. E. Came, *personal communication*) and neutralization tests (H. F. Clark, *unpublished data*). It appears that infection with the amphibian cytoplasmic viruses may be quite common in natural amphibian populations including, besides *R. pipiens*, other species not noted for high tumor incidence.

All strains of amphibian cytoplasmic viruses studied have shown an extraordinary host range in cell cultures incubated at 23 or 30 C. We found no cell culture system of vertebrate origin to be insusceptible. In parallel studies utilizing a representative selection of mammalian and avian viruses inoculated into the same types of cells incubated at 36 C, we have found no other virus to have a host range which approaches the widespread growth capability of the amphibian cytoplasmic virus agents (H. F. Clark, *unpublished data*). In view of their extensive host range in vitro, it has been surprisingly difficult to find a

suitable animal host for study of growth of these viruses in vivo. Propagation of the viruses in chick embryos incubated at 30 C provides a useful source of high-titered virus. However, this is an unnatural host system in that the unphysiologically low temperature causes uninoculated embryos to die after an incubation period of 7 to 8 days, only slightly longer than that required for virus-specific deaths (4 to 6 days).

No replication of the virus in adult *R. pipiens* has been detected. It is not known whether this resistance is species- or age-specific, or whether it is the consequence of an immune response to a commonly encountered infection. An essentially all-or-none difference in susceptibility to virus infection among individual adult newts suggests that in this species resistance may be acquired as the result of infection in part of the population. Further evidence for this hypothesis has been obtained by the isolation of viruses apparently similar to the frog cytoplasmic viruses directly from adult newts. Unlike adults, newts in the immature red eft stage uniformly support growth of frog virus. Red efts may provide a useful system for the study of in vivo growth.

A single isolate, LT-2, differed from the others in producing very small plaques in TH-1A, TH-1B_2, and BHK cell culture, while producing larger plaques in several other types of cells. Rafferty described the production of small plaques, in frog cells only, by FV-3 (18); however, in the present study, FV-3 produced large plaques in TH-1. Plaque-size variation (restricted to certain host-cell culture systems) is the only means so far described for distinguishing any of the isolates of amphibian cytoplasmic viruses. Such plaque markers, if stable, may prove to be useful in genetic studies or in tracing in vivo behavior of these viruses in amphibian hosts, in view of the apparently frequent natural infection of amphibians with similar virus strains.

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