INFECTION AND IMMUNITY, Apr. 1972, p. 559-569
Vol. 5, No. 4
Copyright © 1972 American Society for Microbiology
Printed in U.S.A.

# Iguana Virus, a Herpes-Like Virus Isolated from Cultured Cells of a Lizard, *Iguana iguana*

H. FRED CLARK[1] AND DAVID T. KARZON[2]

*Departments of Pediatrics and Microbiology, School of Medicine, State University of New York, Buffalo, New York 14203*

Received for publication 8 November 1971

An agent cytopathic for *Terrapene* and *Iguana* cell cultures was isolated from spontaneously degenerating cell cultures prepared from a green iguana (*Iguana iguana*). The agent, designated iguana virus, caused a cytopathic effect (CPE) of a giant cell type, with eosinophilic inclusions commonly observed within giant cell nuclei. Incubation temperature had a marked effect on CPE and on virus release from infected cells. Within the range of 23 to 36 C, low temperatures favored CPE characterized by cytolysis and small giant cell formation, and significant virus release was observed. At warmer temperatures, a purely syncytial type of CPE and total absence of released virus were noted. A unique type of hexagonal eosinophilic cytoplasmic inclusion was observed within syncytia of infected *Terrapene* cell cultures incubated at 36 C. In vivo studies revealed no evidence of pathogenicity of iguana virus for suckling mice, embryonated hen's eggs, or several species of reptiles and amphibians. Inoculation of iguana virus into young iguanas consistently caused infection that was "unmasked" only when cell cultures were prepared directly from the infected animal. Filtration studies revealed a virion size of >100 nm and <220 nm. Iguana virus is ether-sensitive and, as presumptively indicated by studies of inhibition by bromodeoxyuridine, possesses a deoxyribonucleic type of nucleic acid. The virus characteristics described, as well as electron microscopy observations described in a separate report, indicate that iguana virus is a member of the herpesvirus group.

The application of modern methods to the study of viruses of poikilothermic vertebrates became feasible following the description by Wolf and Dunbar (39) of methods for culturing fish cells in vitro. Development of fish cell culture was rapidly followed by the isolation of several economically important virus pathogens of fish (22, 40, 42). Subsequently, because of interest in the renal adenocarcinoma enzootic in some populations of leopard frogs (28), several cell culture systems of anuran origin were also developed (13, 33) which in turn led to the isolation of several amphibian viruses [reviewed by Granoff (16)]. We have recently investigated methods for the culture of reptilian cells in vitro in hope of developing cell culture systems useful for isolating viruses from this third major group of poikilothermic vertebrates (4, 7). In the course of these studies, a virus, designated "iguana virus," was isolated from green iguana (*Iguana iguana*) cell cultures which had undergone a spontaneous cytopathic effect (H. F. Clark, R. F. Zeigel, F. B. Fabian, and D. T. Karzon. Bacteriol. Proc., p. 149-150, 1968). Some basic biological and physicochemical properties of this agent are given in this report. The fine structural features of the agent, as revealed by electron microscopy examination, are described in a companion paper (44).

[1] Present address: The Wistar Institute, 36th Street at Spruce, Philadelphia, Pa. 19104.

[2] Present address: Department of Pediatrics, School of Medicine, Vanderbilt University, Nashville, Tenn. 37203.

## MATERIALS AND METHODS

**Poikilothermic vertebrate cell cultures.** The methods for propagation of iguana cell lines, *Gekko* cell lines, and cell lines derived from the box turtle *Terrapene carolina* and the sidenecked turtle *Podocnemis unifilis* have been described (4, 7). Cell lines VSW, derived from a tumor-bearing Russell's viper (*Vipera russelli*) (43), and VH2, derived from a normal Russell's viper (H. F. Clark, M. M. Cohen, and P. D. Lunger. In vitro 6:376, 1971) were cultivated by similar methods. In brief, these reptilian cell lines were grown on glass or plastic surfaces in Eagle's basal medium containing 10% fetal calf serum (BME FCS10) and subcultivated using 0.25% trypsin or 0.02% Versene solution. Incubation temperatures were: *Iguana* cell

line IgH2, 36 C; *Terrapene* cell lines, TH1, TH4, and TH5, 23 C; and all other reptile cell lines, 30 C. Primary reptile kidney cell cultures were prepared by the method of Shindarov (34) and incubated at 30 C. Anuran cell lines RPH67.132 (*Rana pipiens*) and BA68.1 (*Bufo americanus*) (13) were kindly provided by Jerry Freed, Institute for Cancer Research, Fox Chase, Pa., and were propagated according to techniques described by Freed (13) at an incubation temperature of 23 C, in diluted L-15 medium described by Balls and Ruben (1).

Fish cell lines derived from the fathead minnow (FHM) (18) and the bluegill (BF) (41) were propagated in Eagle's minimal essential medium (MEM) with 10% FCS at a temperature of 23 C. Mammalian cell lines WISH, Hep-2, BSC, and green monkey kidney (GMK) were cultivated by standard procedures in MEM FCS10. The methods for preparation of primary monkey kidney and primary whole chick embryo cell cultures have been previously described (3, 23).

**Virus assay methods.** Infectivity titers were determined by inoculating 0.1 ml of 10-fold dilutions of virus in phosphate-buffered saline (0.1 M $PO_4$, *p*H 7.4; PBS) into groups of three or more tubes of cell culture per dilution. Cell culture tubes were examined for cytopathic effect (CPE) daily, and the tissue culture infectivity 50% end-point dose ($TCID_{50}$) was determined by the method of Reed and Muench. Because agar and "agarose" are toxic for many reptilian cells, all plaque assays were performed by the starch gel overlay technique of De Maeyer and Schonne (11) as modified by Lehane et al. (26).

**Poikilothermic vertebrates.** Young iguanas used for virus infectivity studies were purchased from C. F. McClung, La Place, La. Other poikilothermic vertebrates were obtained from the Buffalo Zoo or were collected by the authors.

**Serological studies: antisera.** Rabbits immunized against herpesviruses were given an initial dose of 1.0 ml of antigen via the intramuscular or intravenous route followed by 7 to 10 weekly inoculations of 1.0 ml of equal parts of antigen and complete Freund's adjuvant administered intradermally. Test sera were collected 2 weeks after the final inoculation. The antigens used were: (i) iguana virus propagated in IgH-2 cells, titer ca. $10^{5.5}$ $TCID_{50}$/ml; (ii) Lucké *Rana pipiens* adenocarcinoma herpesvirus, a 10% suspension in PBS of a tumor exhibiting massive involvement with intranuclear inclusions, kindly supplied by Keen Rafferty, Johns Hopkins University; and (iii) avian laryngotracheitis virus (ALTV), a 10% suspension in PBS of infected chick embryo chorioallantoic membranes (CAM). A similar regimen was employed for the preparation of antiserum to the herpesvirus of cobra venom described by Monroe et al. (31). Lyophilized cobra (*Naja n. naja*) venom was kindly supplied by B. D. Ashley, Fort Knox, Ky. The presence of herpesvirus was detected by electron microscopy examination of a suspension of this venom in PBS performed by Robert Zeigel, Roswell Park Memorial Institute, Buffalo, N.Y. The venom dose was gradually increased from an initial concentration of 0.0125 μg/ml to a final concentration given at the 8th to 10th inoculation of 10.0 μg/ml, without ill effect. The preparation of antiserum to herpes simplex virus (HSV) strain HF has been previously described (27). Rabbit antiserum titers to ALTV (CAM-plaque neutralization test) and HSV were 1:100 and > 1:80, respectively. Assay systems for antibody to the Lucké herpesvirus or the cobra venom virus are not currently available.

Antiserum to simian herpesviruses SA-8 and herpes tamarinus were furnished by Lidia Martos, Flow Laboratories, Rockville, Md. Immune serum to monkey B virus was obtained from the Research Reference Reagents Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Antisera to infectious bovine rhinotracheitis, pseudorabies, and equine rhinopneumonitis viruses were kindly supplied by L. E. Carmichael, Veterinary Virus Research Institute, Ithaca, N.Y. Hyperimmune antisera to Burkitt lymphoma cell line P-3 antigen (12) were a gift from Mary Fink, National Cancer Institute; human antisera reactive by indirect immunofluorescent test with a Burkitt lymphoma cell line (19) were a gift from James Blakeslee, Roswell Park Memorial Institute, Buffalo, N.Y.

**Serum neutralization tests.** All sera were heat-inactivated for 30 min at 56 C. Serum-virus mixtures were incubated at 37 C for 30 min. Twofold serum dilutions were incubated with 30 $TCID_{50}$ (for cell culture tube assay) or 50 to 100 plaque-forming units (PFU) of virus per 0.1 ml. In tube tests, 0.2 ml of serum-virus mixture was inoculated into each of three tubes of IgH-2 cell culture for each serum dilution. Tubes were incubated at 30 C and observed daily for 21 days. For plaque assay, 0.2 ml of serum-virus mixture was adsorbed onto duplicate monolayer cultures of two IgH cells in 30-ml plastic flasks. Starch gel overlay was added, and plaques were stained with neutral red and counted after 12 to 14 days.

## RESULTS

**Isolation of iguana virus.** An adult (18-inch, ca. 46-cm) male green iguana obtained from a community lizard and turtle cage at the Buffalo Zoo was sacrificed on February 12, 1965. Minced tissue explant cultures were prepared from the heart, kidneys, liver, and spleen and incubated at 30 or 23 C. Mixed outgrowth of epithelioid and fibroblastic cells commenced in all of the 30 C cultures within 2 to 10 days. Significant cell growth was not obtained at 23 C.

Cell degeneration characterized by giant cell formation was first observed in primary liver cell cultures on the 13th day after explantation. Subsequently similar CPE developed spontaneously in primary spleen (onset day 17), kidney (onset day 21), and heart (onset day 30) cell cultures. In heart cell cultures split 1:2 at approximately 10-day intervals, giant cell CPE first appeared during the fifth passage (47th day). The spontaneous CPE eventually progressed to cause death of all cells in affected cultures.

Supernatant fluids of infected iguana cell cultures were inoculated undiluted into cell cultures of the turtle cell line TH1 (7) and incubated at 23 or 30 C. CPE of a giant cell type was consistently induced in inoculated TH1 cultures, appearing 2 to 10 days after inoculation, earlier at 30 C than at 23 C. The cytopathic agent was named "iguana virus."

Iguana virus was serially passaged, with CPE, in TH1 cells using harvested frozen-thawed whole cultures inoculated onto cell monolayers incubated at 30 C. The efficiency of passage was enhanced when infected cell materials were concentrated by harvesting the cells in 0.2 to 0.1 of the normal volume of growth medium. Such concentrated stocks contained approximately $10^{4.5}$ $TCID_{50}$/0.1 ml. When cell lines of iguana origin became available (4), virus stocks of similar titer were satisfactorily prepared without concentration at harvest. Virus propagated in the cell lines, *Iguana* heart 2 (IgH2) or *Iguana* kidney 17 (IgK17), following 10 initial passages in TH1 cells, was utilized for most experiments (*see below*).

**Reisolation of iguana virus.** Explant cultures prepared from the kidneys and lungs of a second adult (26-inch, ca. 66-cm) male green iguana obtained from the community lizard cage at the Buffalo Zoo on August 9, 1966, spontaneously developed iguana virus-like giant cell CPE within 20 to 30 days. Inoculation of TH1 cell cultures with supernatant fluids from affected cultures led to development of similar iguana virus CPE, but the cytopathic agent was lost in the course of subsequent serial passages. Interestingly, cell cultures prepared from other organs of this animal remained free of CPE; heart cultures were the source of a cell line (IgH2) (4) still in continuous cultivation after more than 5 years and 140 passages.

Virus infection could not be demonstrated in an immature green iguana placed as a "sentinel" in the community lizard cage on September 14, 1966, and sacrificed 63 days later for the preparation of organ explant cell cultures.

**Cultivation of virus in vitro.** Various cell lines of homeothermic and poikilothermic vertebrate origin were tested for susceptibility to iguana virus CPE at an incubation temperature of 30 C. Cell cultures in tubes were inoculated with virus doses of $\geq 10^{4.5}$ $TCID_{50}$ and observed daily for at least 15 days. Progressive CPE of giant cell type was induced in iguana cell lines IgH2, IgVA, IgVB, and IgK17, and in primary kidney cell culture, as well as in box turtle cell lines TH1, TH4, TH4W, and TH5. Limited CPE of giant cell type that could not be serially passaged was induced in *Gekko* cell lines GH1, GH2, and GL1, and in *Caiman* and *Python* primary kidney cell cultures. No CPE was induced in the box turtle cell line TS5, in the sidenecked turtle cell line PH1, or in *Vipera* cell lines VSW and VH. Other cell types refractory to iguana virus infection were the fish cell lines FHM and BF, the amphibian cell lines RPH67.132 (*Rana pipiens*) and BA68.1 (*Bufo americanus*), chick embryo fibroblasts, primary rhesus monkey kidney cells, and the mammalian cell lines WISH, Hep-2, GMK, and BSC.

Iguana virus CPE progressed at similar rates at 30 and 36 C, but was delayed at 23 C. The onset of CPE occurred within 48 hr at the warmer temperatures in cultures inoculated at a multiplicity of $\geq 0.1$ and titration end points were obtained in 7 to 10 days. However, at 23 C, end points were not attained until 17 to 20 days of incubation, and the titers detected were approximately 2.0 $\log_{10}$ lower than those obtained at 30 or 36 C.

The type of CPE induced by iguana virus was also affected by incubation temperature. At 36 C in either *Iguana* or *Terrapene* cells the CPE consisted entirely of irregularly shaped syncytia containing a few to several hundred nuclei (Fig. 1A, B, and C). In cells incubated at 30 or 23 C, the predominant CPE was more cell-destructive, consisting of pycnosis and cytoplasmic retraction of individual cells accompanied by the formation of compact giant cells containing 5 to 10 nuclei and greatly condensed cytoplasm (Fig. 1D and E). The occasional syncytia observed tended to be more highly organized than those formed at 36 C (Fig. 1F). Eosinophilic nuclear inclusions were observed commonly in multinucleated cells formed at 23 to 36 C in all susceptible cell types (Fig. 1A and D).

A unique feature of iguana virus CPE was the formation of very regularly shaped hexagonal eosinophilic cytoplasmic inclusions 5 to 10 $\mu$m in diameter. These were observed only within syncytia formed in *Terrapene* cells at 36 C, but they were numerous under these conditions. These inclusions appeared first in a paranuclear position (Fig. 1B), but as infection progressed they became distributed throughout the syncytial cytoplasm (Fig. 1C). Similar inclusions have not been observed in uninfected *Terrapene* cells, nor in those infected with such herpesviruses as herpes simplex, pseudorabies, or avian laryngotracheitis virus. The composition of these unusual inclusions has not been determined.

Plaque assay of iguana virus could be performed in *Iguana* or *Terrapene* cell monolayers incubated at 30 or 36 C, using starch gel overlay. Plaques reached a size (2 to 3 mm) practical for

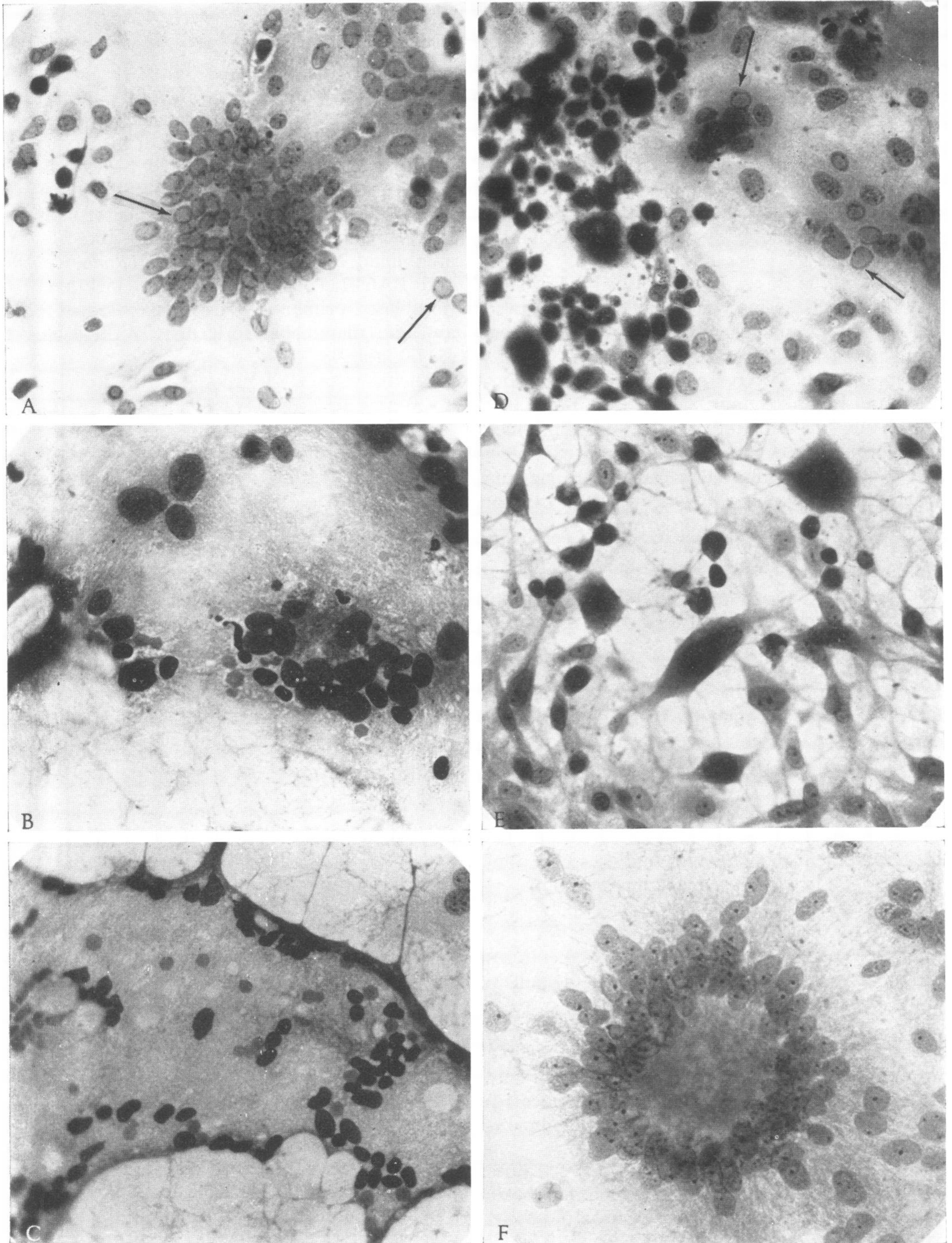


FIG. 1. *Cytopathic effect induced by iguana virus. IgH2 cells were infected at a multiplicity of infection (MOI) of approximately 0.001, and TH1 cells were infected at an MOI of 0.01. Cells were stained with hematoxylin and eosin and photographed at 950× magnification. A, IgH2 cells 10 days after infection at 36 C. CPE is syncytial and intranuclear inclusions (arrows) are prominent. B, TH1 cells 6 days after infection at 36 C, exhibiting syncytia with paranuclear hexagonal cytoplasmic inclusions. C, TH1 cells 8 days after infection at 36 C, old retracting syncytium with hexagonal inclusions scattered in the cytoplasm. D, IgH2 cells 10 days after infection at 30 C. Destructive CPE, characterized by individually rounded cells and small (less than 10 nuclei) dense giant cells, is predominant, but syncytia containing intranuclear inclusions (arrows) are also visible. E, TH1 cells 6 days after infection at 30 C. Individually rounded cells and small dense giant cells are visible. F, TH1 cells 14 days after infection at 23 C. CPE is characterized by highly organized syncytium formation.*

TABLE 1. *Synthesis and release of iguana virus at three different temperatures in cells of the Iguana cell line IgK17*[a]

| Days post-infection | 36 C | | | 30 C | | | 23 C | | |
|---|---|---|---|---|---|---|---|---|---|
| | CAV[b] | RV | CPE[c] | CAV | RV | CPE | CAV | RV | CPE |
| 2 | $1.2 \times 10^4$ | $<1.0 \times 10^0$ | + | $1.3 \times 10^4$ | $1.0 \times 10^1$ | + | $5.0 \times 10^1$ | $<1.0 \times 10^0$ | − |
| 3 | $4.8 \times 10^4$ | $<1.0 \times 10^0$ | + | $8.0 \times 10^4$ | $5.6 \times 10^2$ | + | ND | ND | − |
| 6 | $2.3 \times 10^5$ | $4.0 \times 10^0$ | ++ | ND[d] | ND | +/++ | ND | ND | − |
| 7 | ND | ND | ++ | $2.0 \times 10^6$ | $1.2 \times 10^4$ | ++ | ND | ND | + |
| 10 | $6.8 \times 10^6$ | $<1.0 \times 10^0$ | ++++ | $>1.1 \times 10^7$ | $1.3 \times 10^5$ | ++++ | $1.9 \times 10^5$ | $8.4 \times 10^3$ | ++ |
| 14 | | | | | | ++++ | $1.6 \times 10^6$ | $7.5 \times 10^3$ | ++++ |

[a] Replicate tube cultures infected at a multiplicity of 5.0, with duplicate cultures harvested and pooled at the times indicated.
[b] CAV, cell-associated virus; RV, released virus. All titers are plaque-forming units per milliliter. The base-line (2 hr) CAV yield was $9.8 \times 10^1$.
[c] CPE, cytopathic effect; +, 1 to 25% of the cell monolayer affected; ++, 25 to 50%, etc.
[d] Not done.

counting after 12 to 14 days of incubation. Titers detected were similar at 30 and 36 C in each cell system, but *Iguana* cells gave 10- to 100-fold higher titers than did *Terrapene* cells. Cells of the *Iguana* cell line IgH2 and an incubation temperature of 30 C were selected for routine plaque assay.

**Effect of temperature on virus replication and release.** In view of the poikilothermic nature of its natural host, the effect of incubation temperature on iguana virus replication was studied. Cell cultures of the *Iguana* cell line IgK17 were infected with iguana virus at a multiplicity of infection (MOI) of 5, incubated at 23, 30, or 36 C, and assayed periodically for cell-associated virus (CAV) and released virus (RV) (Table 1).

As indicated by the titers of CAV detected, and the progression of CPE, virus synthesis progressed at approximately equal rates at 30 and 36 C. CPE was first noted on the second day postinfection, and new CAV was detectable at this time. Titers of CAV increased steadily until day 10, when yields of approximately $10^7$ PFU/ml were obtained. At 23 C, virus synthesis progressed more slowly, but by day 14, the titer of CAV was only slightly less than the peak titers obtained at 30 and 36 C.

Incubation temperature had a striking effect on virus release. RV in quantities of roughly 1% those observed to be cell-associated was consistently detected at 23 and 30 C. However, virtually no RV was detected in cell cultures incubated at 36 C. The absence of infectious RV at 36 C cannot be accounted for by the thermal inactivation rate of iguana virus at this temperature (*see below*). Apparently virus release from the syncytia induced by iguana virus at 36 C is much less efficient than is release accompanying the more destructive type of CPE induced at lower temperatures.

A similar pattern of virus growth was obtained in a companion experiment performed in TH1 cell culture, although CPE progressed more slowly and virus yields were 10- to 100-fold lower than in IgK17 cells. Again, virus release was detected from cells incubated at 23 and 30 C, but not at 36 C.

**Growth of iguana virus in vivc.** The effect of inoculation of iguana virus by several different routes into young [about 16-inches (ca. 41 cm) total length] iguanas held at 23 or 30 C was studied. Necropsies were made of animals dead within 15 days after inoculation, and representative organ suspensions were inoculated into TH1 cells incubated at 30 C. Surviving animals were sacrificed on day 15. Suspensions prepared from the organs of the sacrificed animals were inoculated into TH1 cells, and fragments of their minced heart and liver tissues were explanted for cell outgrowth at 30 C in BME FCS10. The results are given in Table 2.

Seven of 12 virus-inoculated iguanas died during the 15-day observation period, whereas only 1 of 6 control animals died. However, no consistent pattern of visceral lesions was observed at necropsy (histopathological studies were not performed). This, coupled with the facts that deaths occurred at intervals scattered throughout the 15-day observation period and that virus was never recovered from organ suspensions of dead animals, suggested that the deaths may not have been causally related to the virus infection.

Attempts to recover virus from triturated organ suspensions of animals sacrificed for viral studies were also unsuccessful, with the exception of one animal subcutaneously inoculated with virus. Virus in low titer ($<10^{3.0}$ $TCID_{50}$/g) was recovered only from the spleen of this animal. Attempts to recover viruses by direct outgrowth of liver and kidney cells in vitro were more successful, as four out of five virus-inoculated animals yielded virus

TABLE 2. *Infection of iguanas with iguana virus*

| Iguana no. | Temperature (C) | Inoculum and route[a] | Disposition of animal[b] | Virus recovery from organ suspension[c] | | | | | | Virus recovery from cell outgrowth[d] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | L | K | S | Bl | Br | G | L | K |
| | | Virus | | | | | | | | | |
| 3 | 30 | 0.25 ml, sc | d, Day 2 | – | – | – | ND[e] | – | ND | | |
| 4 | 30 | 0.25 ml, sc | s, Day 15 | – | – | + | – | – | – | + | + |
| 5 | 23 | 0.25 ml, sc | d, Day 7 | – | – | – | – | – | – | | |
| 6 | 23 | 0.25 ml, sc | d, Day 14 | – | – | – | ND | – | – | | |
| 7 | 30 | 0.03 ml, ic | d, Day 5 | – | – | – | ND | – | – | | |
| 8 | 30 | 0.03 ml, ic | s, Day 15 | – | – | – | – | – | – | + | + |
| 9 | 23 | 0.03 ml, ic | d, Day 5 | – | – | – | ND | ND | – | | |
| 10 | 23 | 0.03 ml, ic | s, Day 15 | – | ND | – | – | – | – | + | + |
| 11 | 30 | 0.25 ml, oral | d, Day 14 | – | – | – | ND | – | – | | |
| 12 | 30 | 0.25 ml, oral | s, Day 15 | – | – | – | – | – | – | + | + |
| 13 | 23 | 0.25 ml, oral | d, Day 3 | ND | – | – | ND | – | – | | |
| 14 | 23 | 0.25 ml, oral | s, Day 15 | ND | – | ND | – | – | – | – | – |
| | | BME CS10 | | | | | | | | | |
| 15 | 30 | 0.25 ml, oral | d, Day 11 | – | – | – | ND | – | – | | |
| 16 | 30 | 0.25 ml, sc | s, Day 15 | – | – | – | – | – | – | – | – |
| 17 | 30 | 0.25 ml, ic | s, Day 15 | – | – | – | – | – | – | – | – |
| 18 | 30 | 0.25 ml, oral | s, Day 15 | – | – | – | – | – | – | – | – |
| 19 | 23 | 0.25 ml, sc | s, Day 15 | – | – | – | – | – | – | – | – |
| 20 | 23 | 0.03 ml, ic | s, Day 15 | – | – | – | – | – | – | – | – |

[a] Virus, iguana virus-infected TH1 cell culture suspension (cell-free); titer, $10^{5.5}$ TCID 50/ml. BME CS10 (Eagle's basal medium plus 10% calf serum) is normal growth medium of TH1 cells, uninfected. sc, Subcutaneous; ic, intracerebral.

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

[c] Virus recovery as indicated by induction of typical cytopathic effect (CPE) in TH1 cell cultures inoculated with 0.1 ml of 10% organ suspension and observed during 21 days of incubation at 30 C. L, liver; K, kidney; S, spleen; Bl, blood; Br, brain; G, gut.

[d] Virus recovery as indicated by appearance of typical CPE in cell outgrowth from organ fragments explanted in BME FCS10 at 30 C.

[e] Not done.

from both the liver and kidney by this technique. The four positive animals included individuals inoculated by the subcutaneous, intracerebral, and oral routes and maintained at both 23 and 30 C.

Each of the virus-positive liver cell cultures developed a typical giant cell type of CPE between 8 and 10 days after explantation, while 21 to 28 days were required for detection of virus in positive kidney cell cultures. Liver and kidney cell cultures prepared from the single virus-inoculated animal from which virus could not be recovered remained normal during three passages and 100 days of observation. Cell cultures derived from all control animals were negative for CPE during 30 to 70 days of observation.

Further attempts were made to infect several other species of reptiles and two species of anuran amphibians (Table 3). The animals were inoculated parenterally with high doses of virus, sacrificed after 14 to 16 days of holding at 23 C, and examined for infection by culture in vitro of organ explants.

Virus was recovered from the single inoculated Tokay gecko by outgrowth of its host cells in vitro (but not by inoculation of liver or kidney suspensions into TH1 cell cultures). Virus was not recovered from nine green anoles, a species more closely related to the iguana (within the family *Iguanidae*). One of two virus-inoculated box turtles yielded virus by direct spleen cell outgrowth, although no virus was recovered from triturated spleen, kidney, or heart suspensions from either turtle. Infection could not be demonstrated in slider turtles, in three species of snakes tested, in the spectacled caiman, or in the anurans—the leopard frog and the American toad.

Iguana virus did not cause overt disease in newborn CFW mice inoculated via the intracerebral, intraperitoneal, or subcutaneous routes.

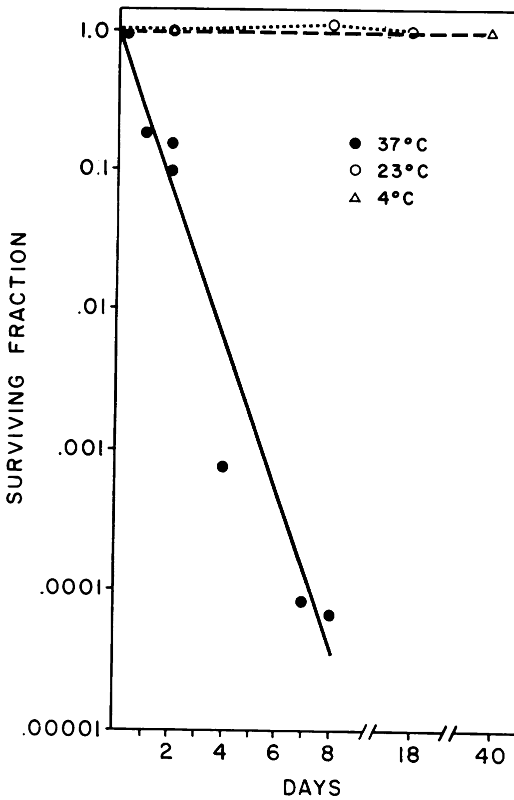


FIG. 2. *Thermal inactivation of iguana virus suspended in BME CS10 at 36, 23, and 4 C.*

Virus was not recovered from the brains of mice sacrificed 7 days after inoculation. Iguana virus did not cause visible lesions during two passages on the CAM of embryonated hen's eggs incubated at 30 C, and virus was not recovered from the CAM.

**Physicochemical properties of iguana virus: thermostability.** The thermal inactivation of iguana virus suspended in cell culture growth medium at temperatures of 4, 23, and 37 C is illustrated in Fig. 2. In this medium (containing 10% calf serum), measurable loss of virus titer was not detectable after 18 days of incubation at 23 C or 40 days of incubation at 4 C. At 37 C, virus was inactivated according to straight-line ("one-hit") kinetics, exhibiting a half-life of approximately 13 hr.

**Filterability.** The results of filtration of iguana virus through a graded series of membrane filters (Millipore, Corp.) are presented in Table 4. The iguana virus preparation tested exhibited no loss in titer after passage through a 450-nm filter. A substantial loss of infectivity was observed after filtration through a 220-nm filter, and a 100-mn filter retained the virus quantitatively. The data suggest a particle size less than 220 nm but greater than 100 nm, findings in agreement with electron microscopy observations that the diameter of enveloped viruses ranges from 165 to 230 nm (44).

**Ether sensitivity.** The results of exposure of iguana virus to 20% diethyl ether are given in Table 4. Iguana virus is ether-sensitive.

**Inhibition by BUdR.** To obtain presumptive evidence of the nucleic acid type of iguana virus, the inhibitory effect of 5'bromo-2'deoxyuridine (BUdR) incorporated in starch gel overlay medium on the plaque formation caused by iguana virus, herpes simplex virus [HSV, deoxyribonucleic acid (DNA)-type control virus] and vesicular stomatitis virus [ribonucleic acid (RNA)-type control virus] was ascertained (Table 4). BUdR at a concentration of 20 $\mu$g/ml led to a $10^4$-fold suppression of the iguana virus PFU titer and a similar suppression of HSV plaque formation. The inhibition by BUdR of iguana virus replication was completely reversed in the presence of excess thymidine. The results suggest that iguana virus possesses a DNA-type nucleic acid.

**Serological studies.** Virus neutralization tests employing tube cell culture assays of residual infectivity failed to reveal neutralizing antibody to iguana virus in the sera of hyperimmunized rabbits. However, in tests employing measurement of virus neutralization by determination of plaque reduction, titers as high as 1:256 (50% plaque reduction end point) were demonstrated in such sera.

The plaque reduction-neutralization test was further employed to assay iguana virus antibody activity in representative experimentally infected and uninfected control reptiles and amphibians and in hyperimmune mammalian reference antisera to representative known herpesviruses. No virus-neutralizing antibody (titer <1:4) was detected in the terminal sera (Tables 2 and 3) of any of the virus-inoculated poikilothermic vertebrates tested, including species susceptible and refractory to experimental infection. Assay of complement-fixing (CF) antibody in these animals was not attempted, because of the incompatability of poikilothermic vertebrate antibodies with the reagents of the mammalian CF system (15).

Iguana virus was also not neutralized (titer <1:4) by antisera to the mammalian herpesviruses, herpes simplex, herpes B, herpes tamarinus, simian agent 8, pseudorabies, equine rhinopneumonitis, and infectious bovine rhinotracheitis, or to avian laryngotracheitis virus. Virus-neutralizing activity was not detected in sera of animals hyperimmunized with the P-3 Burkitt lymphoma antigen nor in human sera possessing antibody to

TABLE 3. *Attempted infection of reptiles and amphibians with iguana virus*[a]

| Species | Common name (no.) | Inoculum and route[b] (ml) | Inoculum titer[c] (per ml) | Virus yield from cell outgrowths[d] |
|---|---|---|---|---|
| Reptiles | | | | |
| *Anolis carolinensis* | Green anoles (1–10) | Virus (0.2), ip | $10^{5.8}$ PFU | – (9H, 9S)[e] |
| | (11–15) | IgH cells (0.2), ip | | – (5H, 5S) |
| *Gekko gecko* | Tokay gecko | Virus (0.2), sc | $10^{4.5}$ $TCID_{50}$ | + (H) |
| *Thamnophis sirtalis* | Garter snake (1) | Virus (0.5), ip | $10^{4.5}$ $TCID_{50}$ | – (K, L) |
| | (2) | TH1 cells (0.5), ip | | – (K, L) |
| *Storeria dekayi* | Brown snake (1) | Virus (0.5), ip | $10^{4.5}$ $TCID_{50}$ | – (K, L) |
| | (2) | TH1 cells (0.5), ip | | – (K, L) |
| *Elaphe obsoleta* | Grey rat snake | Virus, (0.5), ip | $10^{4.5}$ $TCID_{50}$ | – (K, S) |
| *Terrapene carolina* | Box turtle (1) | Virus (0.5), ip | $10^{4.7}$ PFU | – (H) |
| | (2) | Virus (0.5), sc | $10^{4.7}$ PFU | + (S) – (K) |
| | (3) | TH1 cells (0.5), ip | | – (K) |
| *Pseudemys scripta* | Slider turtle (1) | Virus (0.5), sc | $10^{4.5}$ $TCID_{50}$ | – (K, L) |
| | (2) | Virus (0.5), sc | $10^{4.5}$ $TCID_{50}$ | – (K, L) |
| | (3) | TH1 cells (0.5), sc | | – (K, L) |
| *Caiman crocodylus* | Spectacled caiman | Virus (0.5), sc | $10^{4.5}$ $TCID_{50}$ | – (K, L) |
| Amphibians | | | | |
| *Rana pipiens* | Leopard frog (1) | Virus (0.5), ip | $10^{4.5}$ $TCID_{50}$ | – (K) |
| | (2) | Virus (0.5), ip | $10^{4.5}$ $TCID_{50}$ | – (K) |
| | (3) | TH1 cells (0.5), ip | | – (K) |
| *Bufo americanus* | American toad (1) | Virus (0.5), ip | $10^{4.5}$ $TCID_{50}$ | – (K) |
| | (2) | TH1 cells (0.5), ip | | – (K) |

[a] Animals were inoculated with frozen-thawed (three times) preparations of virus-infected or normal cells of the cell culture substrate used for virus propagation. All animals were sacrificed for cell culture studies after 14 to 16 days at 23 C.

[b] ip, Intraperitoneal; sc, subcutaneous.

[c] Plaque-forming unit (PFU) titers were determined in IgH cells, $TCID_{50}$ titers were determined in TH1 cells.

[d] Organs yielding viable cell outgrowth, uncontaminated by bacteria, are listed. All were observed for 30 to 60 days. H, heart; S, spleen; K, kidney; L, liver.

[e] A single anole died 10 days after inoculation.

Burkitt lymphoma membrane antigen detected by immunofluorescence. Sera of rabbits hyperimmunized with leopard frog renal adenocarcinoma, which contained herpesvirus and was positive for nuclear inclusions, and with cobra venom containing herpesvirus likewise exhibited no iguana virus-neutralizing activity.

## DISCUSSION

We have described an apparently new virus, designated iguana virus, isolated from a lizard, *Iguana iguana*. Induction by this virus of a syncytial or giant cell type of CPE characterized by the presence of many intranuclear inclusions, filtration data suggesting a virion diameter between 100 and 220 nm, presumptive evidence of a DNA nucleic acid type, and the demonstration of sensitivity of the virus to inactivation by ether all suggest that it belongs to the herpesvirus group. We have presented evidence in a companion report of electron microscopy studies indicating that this agent also exhibits a fine-structural appearance and an intranuclear site of replication typical of herpesviruses (44). Like many mammalian and avian herpesviruses, iguana virus appears to possess a capability for causing latent or inapparent infection consistently in its namesake host and sporadically in other species of reptiles. The isolation of iguana virus from spontaneously degenerating cell cultures prepared from an apparently normal animal is a feature shared with herpesviruses isolated from the domestic turkey (24), the squirrel monkey (29), the African green monkey (35), the guinea pig (2), the dog (36), the horse (21), the tree shrew (R. Mirkovic, W. R. Voss, and M. Benyesh-Melnick, Proc. Int. Congr. Microbiol. **8**:181, 1970), and the cottontail rabbit (20). The fact that iguana virus is distinct from previously described herpesviruses is indicated by its uniquely restricted host range both in vivo and in vitro and by our failure to demonstrate cross-neutralization of iguana virus by antisera to a representative spectrum of known herpesviruses.

The question of whether iguana virus may be a cytomegalovirus cannot be completely resolved at

TABLE 4. *Physical and chemical properties of iguana virus*

| Determination (Millipore)[a] | Virus titer ($TCID_{50}$) |
|---|---|
| Millipore filtration (pore size)[a] | |
| None | 3.5 |
| 450 nm | 3.5 |
| 220 nm | 1.8 |
| 100 nm | <0.8 |
| Ether inactivation (16 hr at 4 C) | |
| Virus + 20% phosphate-buffered saline | 3.5 |
| Virus + 20% ethyl ether | <0.5 |

Inhibition by BUdR[b]

| Virus | Inhibitor | | |
|---|---|---|---|
| | None | BUdR (20 μg/ml) | BUdR (20 μg/ml) + thymidine (200 μg/ml) |
| Iguana | 5.0 | 1.0 | 5.0 |
| Herpes simplex | 6.0 | 2.0 | ND |
| Vesicular stomatitis | 7.9 | 7.8 | ND |

[a] All titers per milliliter. Virus for filtration was frozen-thawed infected cell suspension sonically treated for 20 sec at 2.5 amp with a Bronson LS-75 sonifier and clarified by centrifugation at 500 × g for 10 min.

[b] Data are titers obtained in TH1 cells at 30 C by plaque assay under starch gel overlay incorporating the inhibitors indicated. BUdR, 5'-bromo-2'deoxyuridine. ND, not done.

this time. Iguana virus shares with cytomegaloviruses the properties of relatively narrow host range restriction and the absence of virus release at certain high temperatures but differs from cytomegaloviruses in not causing cytomegaly in the host cell systems tested and in producing significant quantities of released virus at low incubation temperatures.

Iguana virus was the first virus to be isolated from reptiles (H. F. Clark, R. F. Zeigel, F. B. Fabian, and D. T. Karzon, Bacteriol. Proc., p. 149–150, 1968). Subsequently a herpesvirus-like agent has been detected by electron microscopy observations of the venom of a number of species of elapid snakes (31), but has not been isolated. [We have also recently described a C-type virus produced by cells of a cell line derived from the spleen of a tumor-bearing Russell's viper (43)]. Other herpesviruses described from poikilothermic vertebrates include two antigenically distinct herpesviruses associated with the Lucké tumor of the leopard frog (17), a herpesvirus described by electron microscopy observation of fishpox lesions in the carp (38), and a herpesvirus-designated brown bullhead virus isolated from the fish of that name (K. Wolf, *personal communication*). Of these various herpesviruses of poikilothermic vertebrate origin, only iguana virus and the bullhead isolate can be readily propagated in vitro, producing high yields of released virus.

The effect of temperature on viruses of poikilothermic vertebrate origin is of particular interest. Thermostability studies revealed that iguana virus, coming from a "cold-blooded" vertebrate, exhibited a half-life of 13 hr at 37 C under conditions for which we previously determined a half-life of 2.4 hr for herpes simplex virus (9). Iguana virus replicated efficiently at 36 C and is the first poikilothermic vertebrate virus demonstrated to possess such a capability, which is not surprising in view of the fact that it is also the first virus isolated from a tropical poikilotherm.

Incubation temperature had two profound and unexpected effects on the behavior of iguana virus in vitro. Incubation of iguana virus-infected cell cultures at 36 C led to the development of a predominantly syncytial type of CPE with virus remaining nearly completely cell-associated. Cell cultures infected in parallel, but incubated at 30 C, exhibited a CPE characterized by cytolysis and small giant cell formation, accompanied by the release of substantial amounts of virus into the medium. These observations are of special note because differences similar to those observed in comparative studies of iguana virus CPE obtained at 30 and 36 C have repeatedly been utilized as strain markers of herpes simplex and other herpesviruses [reviewed by Nahmias and Dowdle (32)]; furthermore, strains of herpes simplex distinguished by the type of CPE induced have been reported to induce the formation of distinctly different membrane glyco-proteins in infected cells (25). Moreover, the presence or absence of released virus in cells infected in vitro, a mere temperature effect with iguana virus, has been employed as a fundamental criterion for dividing all of the herpesviruses into two distinct classes (30). The possibility that we may be dealing with a mixed population of iguana herpesviruses, with different types of virus predominating at different incubation temperatures, will be tested by further experiments employing clone-purified virus.

We have shown that iguana virus can be readily recovered by direct cell culture techniques from experimentally infected iguanas with inapparent infections. Thus iguana virus in iguanas provides a practical laboratory system for inducing, and recovering virus from, a latent herpesvirus infection in vivo, something not yet achieved in studies of herpes simplex virus. The study of

latent virus infection in a reptile, and the effect of environmental temperature on such an infection, is of special interest because of the recent demonstration by several authors of the capability of garter snakes to act as efficient overwintering hosts of certain arboviruses (14, 37). Virus infection in such snakes was consistently latent at cold temperatures and active at warmer temperatures, as indicated by the presence of viremia.

Iguana virus replicates in cells of a variety of cell lines of *Terrapene* and *Iguana* origin, which possess widely varying temperature optima for growth (4–6, 8). Preliminary observations suggest that by selection of the proper incubation temperature, chronic iguana virus infection can be readily established in certain of these cell lines without inclusion of antiviral serum in the medium. Such systems are difficult to establish in mammalian cells infected with herpes simplex virus (10).

Iguana virus would seem to represent an unusually useful tool for the study of infection, particularly latent infection, in reptiles. Such knowledge is important if we are to determine the role of poikilotherms as real or potential reservoir hosts of mammalian viruses. Further study of such an exotic virus would also seem to be justified on the grounds that enlightened ecological policies must require knowledge of infectious disease processes present in all components of the wildlife population.

## ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant CA-08737 from the National Cancer Institute and by research grant AI-02396 and training grant AI-098 from the National Institute of Allergy and Infectious Diseases.

We gratefully acknowledge the excellent technical assistance of Frances Fabian and Maria-Elvira Soriano.

## LITERATURE CITED

1. Balls, M., and L. N. Ruben. 1966. Cultivation *in vitro* of normal and neoplastic cells of *Xenopus laevis*. Exp. Cell Res. 43:694–695.
2. Bhatt, P. N., D. H. Percy, J. L. Craft, and A. M. Jones. 1971. Isolation and characterization of a herpeslike (Hsiung-Kaplow) virus from guinea pigs. J. Infect. Dis. 123:178–189.
3. Bussell, R. H., and D. T. Karzon. 1959. Cytopathic effect of canine distemper virus in tissue culture. Science 130:1708–1709.
4. Clark, H. F., M. M. Cohen, and D. T. Karzon. 1970. Characterization of reptilian cell lines established at incubation temperatures of 23 to 36°. Proc. Soc. Exp. Biol. Med. 133:1039–1047.
5. Clark, H. F., and L. Diamond. 1971. Comparative studies on the interaction of benzpyrene with cells derived from poikilothermic and homeothermic vertebrates. II. Effect of temperature on benzpyrene metabolism and cell multiplication. J. Cell. Physiol. 77:385–392.
6. Clark, H. F., F. Kaminski, and D. T. Karzon. 1970. Thermoelectrically cooled temperature-gradient apparatus for comparative cell and virus temperature studies. Appl. Microbiol. 19:848–854.
7. Clark, H. F., and D. T. Karzon. 1967. *Terrapene* heart (TH-1), a continuous cell line from the heart of the box turtle *Terrapene carolina*. Exp. Cell Res. 48:263–268.
8. Clark, H. F., and D. T. Karzon. 1967. Acquired tolerance to elevated temperatures in a poikilothermic cell line (*Terrapene* heart, TH-1). Exp. Cell Res. 48:269–275.
9. Clark, H. F., and D. T. Karzon. 1968. Temperature optima of mammalian and amphibian viruses in cell cultures of homeothermic and poikilothermic origin. Arch. Gesamte Virusforsch. 23:270–279.
10. Coleman, V., and E. Jawetz. 1961. A persistent herpes simplex infection in antibody-free cell culture. Virology 13:375–377.
11. De Maeyer, E., and E. Schonne. 1964. Starch gel as an overlay for the plaque assay of animal viruses. Virology 24:13–18.
12. Fink, M. A., and C. A. Cowles. 1968. Use of immunological techniques in the study of human leukemia, p. 155–162. *In* C. J. D. Zarafonetis (ed.), Proceedings of the International Conference on Leukemia-Lymphoma. Lea and Febiger, Philadelphia.
13. Freed, J. J., L. Mezger-Freed, and S. A. Schatz. 1969. Characteristics of cell lines from haploid and diploid anuran embryos, p. 101–111. *In* M. Mizell (ed.), Biology of amphibian tumors. Recent results in cancer research, special supplement. Springer-Verlag, New York.
14. Gebhardt, L. P., G. J. Stanton, D. W. Hill, and G. C. Collett. 1964. Natural overwintering hosts of the virus of western equine encephalitis. N. Engl. J. Med. 271:172–177.
15. Gewurz, H., J. Finstad, L. H. Muschel, and R. A. Good. 1966. Phylogenetic inquiry into the origins of the complement system. p. 105–117. *In* R. T. Smith, P. A. Miescher, and R. A. Good (ed.), Phylogeny of immunity. Univ. of Florida Press, Gainesville.
16. Granoff, A. 1969. Viruses of amphibia. Curr. Top. Microbiol. Immunol. 50:107–137.
17. Gravell, M. 1971. Viruses and renal carcinoma of *Rana pipiens*. X. Comparison of herpes-type viruses associated with Lucké tumor-bearing frogs. Virology 43:730–733.
18. Gravell, M., and R. G. Malsberger. 1965. A permanent cell line from the fathead minnow (*Pimephales promelas*). Ann. N.Y. Acad. Sci. 126:555–565.
19. Hinuma, Y., M. Konn, J. Yamaguchi, D. J. Wudarski, J. R. Blakeslee, Jr., and J. T. Grace, Jr. 1967. Immunofluorescence and herpes-type virus particles in the P3HR-1 Burkitt lymphoma cell line. J. Virol. 1:1045–1051.
20. Hinze, H. C. 1971. New member of the herpesvirus group isolated from wild cottontail rabbits. Infect. Immunity 3:350–354.
21. Hsiung, G. D., H. R. Fischman, C. K. Y. Fong, and R. H. Green. 1969. Characterization of a cytomegalo-like virus isolated from spontaneously degenerated equine kidney kidney cell culture. Proc. Soc. Exp. Biol. Med. 130:80–84.
22. Jensen, M. H. 1965. Research on the virus of Egtved disease. Ann. N. Y. Acad. Sci. 126:422–426.
23. Karzon, D. T., B. F. Pollack, and A. L. Barron. 1959. Phase variation in ECHO virus type 6. Virology 9:564–576.
24. Kawamura, H., D. J. King, Jr., and D. P. Anderson. 1969. A herpesvirus isolated from kidney cell culture of normal turkeys. Avian Dis. 13:853–863.
25. Keller, J. M., P. G. Spear, and B. Roizman. 1970. Proteins specified by herpes simplex virus. III. Viruses differing in their effects on the social behavior of infected cells specify different membrane glycoproteins. Proc. Nat. Acad. Sci. U.S.A. 65:865–871.
26. Lehane, D. E., Jr., H. F. Clark, and D. T. Karzon. 1967. A plaque method for titration of frog viruses using starch gel overlay. Proc. Soc. Exp. Biol. Med. 125:50–54.
27. Lehane, D. E., Jr., H. F. Clark, and D. T. Karzon. 1968. Antigenic relationships among frog viruses demonstrated by the plaque reduction and neutralization kinetics tests. Virology 34:590–595.

28. Lucké, B. 1934. A neoplastic disease of the kidney of the frog, *Rana pipiens*. Amer. J. Cancer **20**:352–379.
29. Melendez, L. V., M. D. Daniel, R. D. Hunt, and F. G. Garcia. 1968. An apparently new herpesvirus from primary kidney cultures of the squirrel monkey (*Saimiri sciureus*). Lab. Anim. Care **18**:374–381.
30. Melnick, J. L., and R. M. McCombs. 1966. Classification and nomenclature of animal viruses. Progr. Med. Virol. **8**:400–409.
31. Monroe, J. H., G. P. Shibley, G. Schidlovsky, T. Nakai, A. F. Howatson, N. W. Wivel, and T. E. O'Connor. 1968. Action of snake venom on Rauscher virus. J. Nat. Cancer Inst. **40**:135–145.
32. Nahmias, A. J., and W. R. Dowdle. 1968. Antigenic and biologic differences in herpesvirus hominis. Progr. Med. Virol. **10**:110–159.
33. Rafferty, K. A. 1965. The cultivation of inclusion-associated viruses from Lucké tumor frogs. Ann. N.Y. Acad. Sci. **126**:3–21.
34. Shindarow, L. 1962. Tissue culture of kidney epithelium of tortoise (*Testudo graeca*). Dokl. Bulgarska Akad. Naukite Sofia) **15**:539–542.
35. Smith, K. O., J. F. Thiel, J. T. Newman, E. Harvey, M.D. Trousdale, W. D. Gehle, and G. Clark. 1969. Cytomegaloviruses as common adventitious contaminants in primary African green monkey kidney cell cultures. J. Nat. Cancer Inst. **42**:489–497.
36. Spertzel, R. O., D. L. Huxsoll, S. J. McConnell, L. N. Binn, and R. H. Yager. 1965. Recovery and characterization of a herpes-like virus from dog kidney cell culture. Proc. Soc. Exp. Biol. Med. **120**:651–655.
37. Thomas, L. A., and C. M. Eklung. 1960. Overwintering of western equine encephalomyelitis virus in experimentally infected garter snakes and transmission to mosquitoes. Proc. Soc. Exp. Biol. Med. **105**:52–55.
38. Wolf, K. 1966. The fish viruses. Adv. Virus Res. **12**:35–101.
39. Wolf, K., and C. E. Dunbar. 1957. Cultivation of adult teleost tissues *in vitro*. Proc. Soc. Exp. Biol. Med. **95**:455–458.
40. Wolf, K., M. Gravell, and R. G. Malsberger. 1966. Lymphocystis virus: isolation and propagation in Centrarchid fish cell lines. Science **151**:1004–1005.
41. Wolf, K., and M. C. Quimby. 1969. Fish cell and tissue culture, p. 253–305. *In* Fish physiology, vol. 3. Academic Press Inc., New York.
42. Wolf, K. S. F. Snieszko, C. E. Dunbar, and E. Pyle. 1960. Virus nature of infectious pancreatic necrosis in trout. Proc. Soc. Exp. Biol. Med. **104**:105–108.
43. Zeigel, R. F., and H. F. Clark. 1969. Electron microscopic observations on a "C"-type virus in cell cultures derived from a tumor-bearing viper. J. Nat. Cancer Inst. **43**:1097–1102.
44. Zeigel, R. F., and H. F. Clark. 1972. Electron microscopy observations of a new herpes-type virus isolated from *Iguana iguana* and propagated in reptilian cells in vitro. Infect. Immunity 5:570–582.