Growth and Attenuation of Rabies Virus in Cell Cultures of Reptilian Origin[1] (36354)

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Rabies has traditionally been known as a disease of "warm-blooded" vertebrates; rabies virus has been successfully propagated in cell cultures derived from widely diverse species of mammals and birds (1, 2). Success achieved in attempts to propagate several other homeothermic vertebrate viruses in reptilian cell culture (3, 4) led us to investigate the possibility of cultivating rabies virus in cells of certain reptilian, amphbian, and piscine cell lines. The relative susceptibility of several such cell lines to rabies virus is described in this report. The ability of the recently discovered (5) rabies-related Ibadan shrew and Lagos bat viruses to replicate in certain reptilian cells was also demonstrated.

The virulence for mice of rabies virus continuously cultivated in viper and gecko cells has been monitored. The attenuation of this virus for mice after passage in these reptilian cells is herein reported. Alteration, in some cases, of plaque morphology and temperature sensitivity is also described.

Materials and Methods. Cell cultures. The trout cell line RTG-2 (6) was kindly supplied to us by Dr. Ken Wolf, Eastern Fish Disease Laboratory, U.S. Public Health Service, Kearneysville, WV. Fathead minnow cell line FHM (7) was purchased from Grand Island Biological Company. Amphibian cell lines RPH67.132 and BA68.1 (8) were generously provided by Dr. Jerry Freed, Institute for Cancer Research, Fox Chase, PA. Reptilian cell lines were established in the author's laboratory.

Detailed procedures for the propagation of each cell line are described elsewhere (4, 6, 7, 9). Fish and reptile cell lines were grown in Eagle's basal medium (medium for fish cells contained 2X concentration of amino acids and vitamins) while amphibian cells were grown in isotonically diluted Leibovitz medium (10). All media were supplemented with 10% fetal bovine serum. Cell lines of viper, iguana, and side-necked turtle origin were grown at 30°, while all other cell lines were normally propagated at 23°. Both stock cultures and virus-infected cell cultures were subcultured by dispersion with a Versene (0.02%) plus trypsin (0.25%) solution and replating at a 1:2 cell dilution.

Virus. Strain CVS (mouse-fixed) rabies virus adapted to hamster kidney cell culture (1) was originally supplied to this laboratory by Dr. R. E. Kissling, Center for Disease Control, U.S. Public Health Service, Atlanta, GA, and was adapted to BHK/21 cell culture and clone-purified by Dr. T. J. Wiktor. Strain ERA rabies virus was recovered from a vial of commercial ERA vaccine (Connaught Laboratories) and similarly adapted to BHK/21 cell culture and cloned. Virus stocks were prepared in BHK/21 cell cultures maintained with Eagle's basal medium containing 0.1% bovine serum albumin and incubated at 33°. Virus plaque assays were performed by the agarose-suspended BHK/21 13S cell technique of Sedwick and Wiktor (11).

Ibadan shrew virus was obtained from Dr. Robert Shope, Yale University School of Medicine, New Haven. Lagos bat virus was supplied by Dr. Fred Murphy, Center for Disease Control, U.S. Public Health Service, Atlanta, GA. With each virus, an original stock obtained from suckling mouse brain suspension was serially propagated in BHK/

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TABLE I. Susceptibility of Poikilothermic Vertebrate Cell Lines to Rabies Virus.[a]

Cell species	Cell line	Virus strain	Maximum rabies virus infection: Immunofluorescent antigen (%)	Maximum rabies virus infection: Released virus (pfu/ml)	Maximum no. of infected passages
Fish					
Bluegill (*Lepomis microchirus*)	BF	CVS	2	2.0×10^3	4
Fathead minnow (*Pimephales promelas*)	FHM	ERA	1	ND[b]	1
Amphibian					
Leopard frog (*Rana pipiens*)	RPH 67.132	CVS	0	9.5×10^1	1
American toad (*Bufo americanus*)	BA 68.1	CVS	0	3.0×10^1	1
Reptile					
Side-necked turtle (*Podocnemis unifilis*)	PH2	CVS	90	1.0×10^4	16[c]
Russell's viper (*Vipera russelli*)	VSW	CVS	100	1.0×10^7	107[d]
	VH2	CVS	10	1.4×10^5	18
Green iguana (*Iguana iguana*)	IgH2	CVS	1	4.3×10^3	2
Tokay gecko (*Gekko gecko*)	GL1	CVS	10	3.4×10^4	64

[a] Cell cultures infected in monolayer at a multiplicity of approximately 1.0 and incubated at 33°; serial passages performed by subculture of viable infected cells.

[b] ND = not determined.

[c] Passages discontinued because of spontaneous degeneration of cell line (infected and control) cultures).

[d] Serial passages still in progress.

21 cells for approximately 10 passages. At this passage level plaques were readily induced in agarose-suspended BHK/21 cells.

Assay of mouse virulence of rabies virus. Mouse pathogenicity measured in terms of 50% mouse lethal doses per plaque-forming unit (MLD_{50}/pfu) was determined by simultaneous inoculation of serial dilutions of virus preparations: (a) onto duplicate plates for plaque assay, and (b) intracerebrally into groups of six 6-week-old ICR mice. The degree of retention of immunogenicity in attenuated virus substrains was determined by challenging all mice surviving inoculation with test virus at 30 days postinoculation. The challenge dose was approximately 100 LD_{50} of CVS virus propagated in mouse brain.

Results. Susceptibility of poikilothermic vertebrate cells to rabies virus infection. The results of a survey of the susceptibility of nine cell lines of cold-blooded vertebrate origin to infection with fixed rabies virus (primarily CVS strain) previously adapted to BHK/21 cell culture are presented in Table I. Cell cultures were infected at a virus multiplicity of approximately 1–2 and examined for the presence of rabies immunofluorescent antigen and infectious released virus at regular intervals. Positive cultures were passaged by serial subculture of viable cells [a technique previously applied successfully to the adaptation of animal-passaged rabies virus to mammalian cell culture (2)] for as long as immunofluorescent rabies antigen or infectious virus production could be detected.

Only minimal infections were induced in two fish cell lines and no infection was observed in the amphibian cells tested. Our original failure to infect the FHM fish cell line is contradicted by the findings of Solis and Mora (12) that HEP strain fixed virus replicates to high titer in those cells and by preliminary data recently obtained in our laboratory (Michalski and Clark, unpublished data) suggesting that CFS virus also may, under certain conditions, be serially propagated in FHM cells, with sustained production of released virus in very low titer.

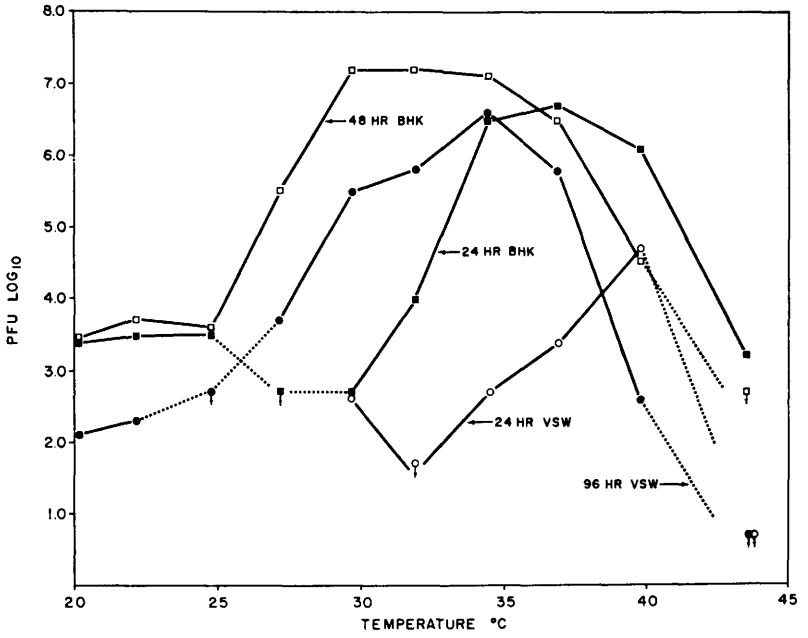

FIG. 1. Released virus titers (pfu/ml) obtained in replicate BHK/21 and VSW cell cultures infected with strain ERA rabies virus and incubated at points along a 20.0 to 43.6° temperature gradient in a temperature gradient incubator.

A wide variation was found in the degree of susceptibility of reptilian cell lines to rabies virus. The lizard cell line IgH2 exhibited only minimal susceptibility, whereas the C-particle-producing viper cell line VSW supported the replication of rabies virus at an efficiency exceeding that of many mammalian cell types.

Virus infection was maintained in cells of the nonvirogenic viper cell line VH2 for 18 passages during which the incidence of cells exhibiting immunofluorescent rabies antigen (IFRA) varied from 1 to 10%, while released virus (RV) titers ranged from $< 5.0 \times 10^0$ to 1.4×10^5 pfu/ml. The VH2 cells spontaneously became virus-free at passage level 19.

Cells of the turtle cell line PH2 were also readily infected. As many as 90% of cells were found to exhibit IFRA at various passage levels but virus replication (or possibly virus release) was inefficient, as the maximum RV titer obtained was only 1.0×10^4 pfu/ml. After 16 serial passages, the rabies-infected PH2 cell line was lost when it spontaneously degenerated at cell passage level 155.

Cytopathic effect was not noted in infected PH2 or VH2 cells, but occasionally infection so retarded cell growth that the addition of uninfected cells was required at the time of subculture. This phenomenon has also been noted previously in the case of rabies virus-infected mammalian cells (2). Virus from passage 10 of both infected VH2 and PH2 cell lines was found to retain normal pathogenicity for adult mice (MLD_{50}/pfu ratio = ca. 1.5).

The effect of prolonged serial passage of CVS rabies virus in the viper cell line VSW and in the lizard cell line GL1, is described in detail below.

Serial passage of strain CVS virus in VSW cells. The VSW cell line was the most susceptible to rabies virus infection of the poikilothermic vertebrate cell types tested. VSW cells were the only reptilian cells in which rabies virus could be serially propagated by passage of infected cell supernatant fluids alone. CVS rabies virus was passaged successfully by transfer of supernatant fluids for 25 passages at an incubation temperature of 33°.

TABLE II. Mouse Virulence and Immunogenicity of CVS Rabies Virus During Serial Passage in Reptile Cells.[a]

Virus	Passage level[b]	Virulence (MLD_{50}/pfu)	Immunogenicity (living virus)[c] (50% protective dose) (pfu)
A. CVS-BHK/21 cell-adapted parental strain		1.0–5.0	NSP
B. CVS-VSW, Expt. 1 (infected medium passage)	20 + 1 BHK	5.0	NSP
	25	1.4	NSP
C. CVS-VSW, Expt. 2 (passage by cell subculture)	27	0.84	NT
	50	0.76	NT
	50 + 1 BHK	1.4	NT
	81[d]	0.018	0.24
		<0.000034	≤10
	83	<0.0013	4.8
	89	<0.00034	3.6
	89 + 2 BHK	<0.000010	10
D. CVS-GL1, Expt. 1 (passage by cell subculture)	15 + 1 BHK	0.77	NT
	30 + 2 BHK	0.46	NT
	40	<0.0059	NT
	58 + 2 BHK	<0.0000024	770
	60 + 2 BHK[d]	<0.0000012	1100
		<0.0000017	14,000
	60 + 3 BHK	0.000012	3300
E. CVS-GL1, Expt. 2 (passage by cell subculture)	5 + 2 BHK[d]	0.24	NSP
		0.22	NSP

[a] Virulence is expressed as the number of 50% lethal doses [for intracerebrally (ic) inoculated adult mice] per plaque-forming units: MLD_{50}/pfu. Immunogenicity is measured by the number of pfu (inoculated ic into adult mice) required to protect 50% of the mice against ic challenge 30 days postinfection with approximately 100 MLD_{50} of CVS virus.

[b] Some test virus was prepared by the indicated number of additional passages in BHK/21 cell culture.

[c] NSP = no survivors of infection protected; NT = not tested.

[d] Results on duplicate determinations performed with one virus stock.

The attempts to accomplish serial virus passage at 25° were unsuccessful, despite the fact that the capacity of rabies virus to replicate at this temperature in mammalian cells has been demonstrated (Clark, H. F., unpublished data).

Measurement of RV titers obtained in VSW and BHK/21 cells incubated at temperatures varying from 20.2 to 43.6° in a temperature gradient incubator (13) (Fig. 1) revealed that replication of rabies virus: (a) occurred first at the highest temperature tolerated (40.2°) by VSW cells, ()b proceeded more slowly at all temperatures in VSW than in BHK/21 cells, and (c) eventually exhibited a temperature response at 4 days in VSW cells that was closely parallel to that observed after 2 days in BHK/21 cells.

Rabies virus yields obtained during serial passage of infected cell fluids at 33° ranged from 2.7×10^4 to 1.7×10^6 pfu/ml and did not increase with continued passage. Virus tested at passage level 25 was found to possess undiminished pathogenicity for adult mice (1.4 MLD_{50}/pfu) ([Table II (B)].

CVS rabies virus infection was also maintained in infected VSW cells passaged by viable cell subculture at approximately weekly intervals for 95 passages at 33° after which the infection was lost. A subline of these CVS virus-infected cells, established by transfer of infected cells from 33 to 30° at

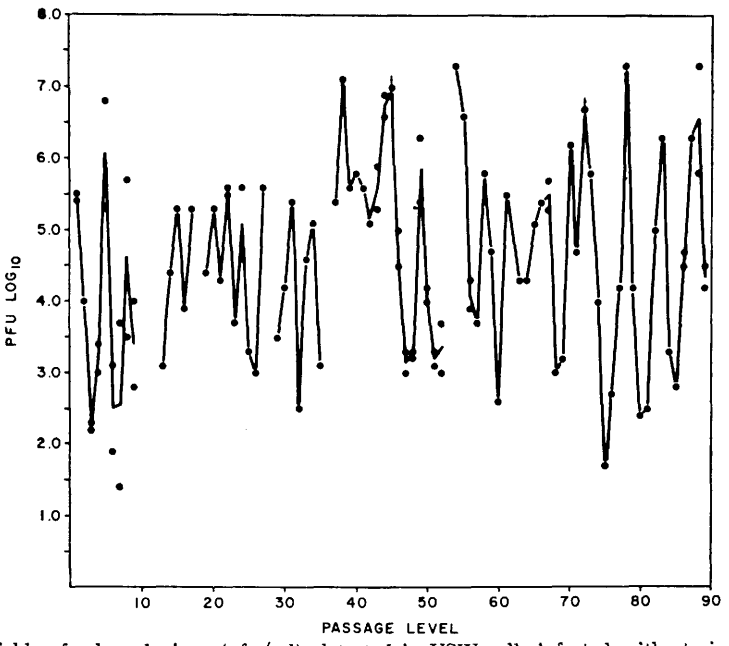

FIG. 2. Yields of released virus (pfu/ml) detected in VSW cells infected with strain CVS rabies virus and serially passaged by subculture of viable infected cells.

rabies virus to date (passage level 107, Sept. 1971).

A cytopathic effect was not noted in virus-infected cells, but the addition of fresh uninfected VSW cells was often necessary at the time of subculture. Virus cytopathogenicity did not apparently increase with serial passage.

RV titers detected during the first 90 serial passages of CVS virus-infected VSW cells are depicted in Fig. 2. Virus yields rose and fell according to an irregular cyclical pattern, with RV titers ranging from low values of less than 1×10^2 pfu/ml to peak values of greater than 1×10^7 pfu/ml. A cyclical pattern of rabies virus production in serially subcultured viable infected mammalian cells has been previously described (14); a more detailed analysis of this phenomenon in mammalian and viper cells will be described elsewhere (T. J. Wiktor, unpublished data).

The virulence and protective potential of rabies virus tested at representative VSW cell passage levels in this subculture series were assayed by intracerebral inoculation of adult mice [Table II (C)]. Normal levels of mouse pathogenicity characteristic of CVS strain rabies virus were detected in assays performed up to and including VSW cell passage level 50. At passage level 81, pathogenicity for adult mice was sharply reduced, and in tests performed at higher passage levels essentially no mouse-lethal potential was detected. Nevertheless, this attenuated rabies virus apparently retained a capacity for infecting mice, as indicated by its potent immunizing capacity. In repeated tests, the 50% protective dose against challenge for adult mice was only 10 or fewer pfu.

Serial passage of strain CVS virus in GL1 cells. The lizard cell line GL1 supported rabies virus replication at a much lower titer than did the VSW cells; serial virus passage could be accomplished only by subculture of viable infected cells (Fig. 3). In an initial series of subcultures, virus infection was maintained for 64 passages at 33°, after which the cells became virus-free. In a second experiment rabies virus infection was maintained for only 11 passages.

RV titers measured during 64 passages of virus-infected GL1 cells never exceeded $10^{5.0}$ pfu/ml and on several occasions dropped

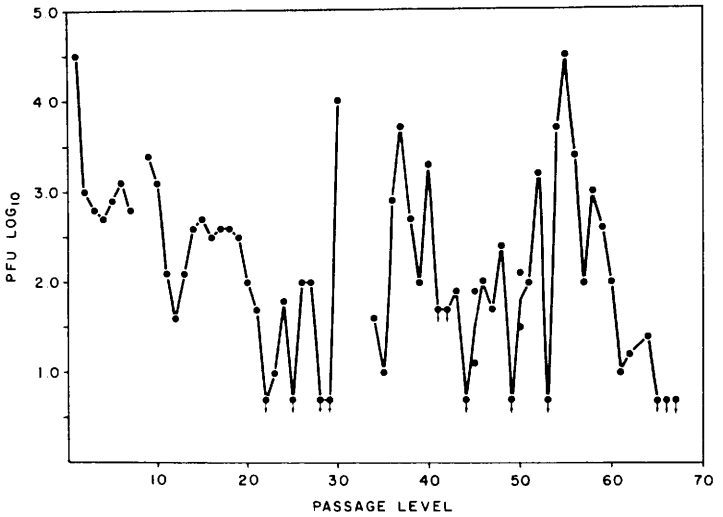

FIG. 3. Yields of released virus (pfu/ml) detected in GL1 cells infected with strain CVS rabies virus and serially passaged by subculture of viable infected cells.

below detectable levels ($< 5.0 \times 10^0$ pfu/ml), while infection was nevertheless maintained. The incidence of virus-infected cells detected by fluorescent antibody examination varied from none observed ($< .1\%$) to 10%. The complete absence of virus in cells tested subsequent to passage 64 was confirmed by: (a) failure to detect infectious virus or IFRA for 12 consecutive cells passages; (b) failure to rescue virus by cocultivation of the GL1 cells with the highly susceptible BHK/21 cells for periods up to 14 days at 35°; (c) failure of the GL1 cells to induce rabies infection or protection against rabies virus challenge in intracerebrally inoculated newborn or adult mice.

In the first series of rabies virus passages, the virus acquired a small plaque-forming marker by passage 25, which was retained during subsequent passages in GL1 or BHK cells. In the second GL1 cell passage series, virus capable of producing only minute plaques appeared within 5 passages.

The mouse-pathogenic properties of GL1 cell-propagated rabies virus tested at selected passage levels are listed in Table II, D and E. Because of the low virus yields characteristic of rabies virus-infected GL1 cells, it was usually necessary to cultivate the GL1 cell-propagated virus for 1 to 3 additional passages in BHK/21 cells to obtain virus in concentrations useful for *in vivo* testing. In the GL1 cell passage series 1, CVS virus retained near-normal mouse pathogenicity through passage level 30 (after the appearance of the small plaque character). At the 40th and subsequent passage levels, the virus was nearly completely nonpathogenic for adult mice. However, the GL1 cell-attenuated virus was not as effective an immunizing agent as the VSW cell-attenuated variant described above; the 50% protective dose of this virus necessary to protect mice against challenge ranged from 770 to 14,000 pfu in different experiments. The small-plaque rabies virus variant which appeared after only 5 serial passages in GL1 cell passage series 2, retained near-normal virulence for adult mice.

Propagation of rabies-related Ibadan shrew and Lagos bat viruses in VSW and GL1 cells. Two rhabdoviruses isolated from Nigerian wildlife and designated Lagos bat virus and Ibadan shrew virus, respectively, have recently been reported to bear an antigenic relationship to rabies virus (5), necessitating a reevaluation of the traditional dogma that rabies virus is antigenically unique. The Lagos and Ibadan viruses have been adapted to BHK/21 cell culture in this laboratory, and three cloned substrains differing in plaque morphology [large (L), medium

(M), or small (S)] have been selected from BHK/21 cell-adapted virus stocks of each, by Dr. T. J. Wiktor (unpublished data). We have tested the capacity of each of these 6 virus substrains for replication in the reptilian VSW and GL1 cell lines.

Cell cultures were inoculated with virus at a multiplicity of infection of approximately 1.0, incubated at 30°, and passaged by subculture of viable cells at 7-day intervals. VSW cells uniformly supported replication of each of the six viruses, and infection was maintained during serial passage. Released virus yields obtained after the first passage were similar to those obtained in BHK/21 cells, varying from 1.6×10^7 to 5.0×10^7 pfu/ml with Lagos virus and from 1.4×10^5 to 5.0×10^7 pfu/ml in the case of Ibadan virus. Two substrains each of Lagos and Ibadan virus were serially cultivated for 10 to 15 passages in VSW cells. Virus yields in all cases varied irregularly; titers as low as 5.0×10^0 and as high as those obtained at the first passage level were obtained during the course of the serial passage. As in the case of rabies virus, cell growth was frequently inhibited; but no increase in cytopathogenicity or alteration of plaque morphology was noted.

First passage yields of Lagos and Ibadan viruses in GL1 cells ranged from 1.3×10^5 to 1.4×10^6 pfu/ml, slightly less than the yields obtainable in BHK/21 cells. None of the three substrains of Ibadan virus could be successfully maintained in serial passage in GL1 cells; each Ibadan virus-infected cell series was virus-free by the third passage. On the other hand, each of the three substrains of Lagos virus was maintained in serial passage in GL1 cells for at least 10 passages, yielding released virus in titers ranging from 1.5×10^1 to 3.0×10^5 pfu/ml. No alteration in plaque morphology or pathogenicity for mice resulting from GL1 cell passage of this duration was observed.

Discussion. A survey of poikilothermic vertebrate cell lines has disclosed a surprising degree of variability in susceptibility to infection by rabies virus; the viper cell line VSW supports rabies virus replication with an efficiency surpassing most mammalian cell systems, while the lizard cell line IgH2 and amphibian cell lines RPH67.132 and BA68.1 are the only vertebrate cell lines that we have found to be nearly completely refractory to infection. The reptilian cell lines VSW and GL1, which supported rabies virus replication for the most prolonged periods, both spontaneously produce C-type virus particles, the former in copious quantities (15) and the latter in very small number (Lunger, P. D., and Clark, H. F., unpublished data). Simultaneous maturation by budding from the VSW cell plasma membrane of rabies virus and C-type virus, in near proximity, has been observed by the electron microscopic observation (Lunger and Clark, unpublished data). The role of these C-type viruses indigenous to certain reptiles in determining the susceptibility of the host cell to superinfection with rabies virus is being further investigated.

While the susceptibility of certain fish and reptilian cell lines to infection with a variety of mammalian viruses has been previously reported (3, 4, 6, 11, 16), we are aware of only a single attempt to assess the effect of serial passage in poikilothermic vertebrate cells on virus virulence. In that limited study, Pudney and Varma (17) reported the retention of normal virulence for mice of a strain of louping ill virus following 11 serial passages in cell cultures derived from the frog *Rana temporaria*.

We have demonstrated the development of complete attenuation for adult mice of CVS rabies virus after 81 serial passages in viper (VSW) cells and 40 serial passages in gecko (GL1) cells. (As in the case of many attenuated rabies virus strains, pathogenicity for intracerebrally-inoculated newborn mice was retained.) The VSW cell-attenuated virus has characteristics that might merit its consideration as a live vaccine strain. It is almost completely apathogenic for adult mice, yet the dose required for immunization via the intracerebral route is only 10 or fewer pfu. A definitive evaluation of the usefulness of this rabies strain will require additional testing of its immunogenicity when administered via peripheral inoculation routes. The presence of C-type virus in VSW cells renders the virus

strain unsuitable for human vaccine use, by current standards, but the principle that mammalian virus may be attenuated while retaining a high degree of immunogenicity by passage in reptilian cells, has been established. Further studies involving attempts to attenuate rabies virus in nonvirogenic reptilian cell types appear to be justified.

In addition to becoming further attenuated, rabies virus subjected to prolonged passage in VSW or GL1 cells also becomes incapable of replication at 40.5° (rct/40.5—marker, Clark, H. F., unpublished data). In two series of GL1 cell passages, rabies virus acquired a small-plaque marker within 25 and only 5 passages, respectively. The latter small-plaque variant is also a temperature-sensitive mutant. Conceivably, passage of the mammalian rabies virus in the phylogenetically distant GL1 cell type may lead to induction or selection of virus mutations with an efficiency superior to that observed in cells more closely related to the normal *in vivo* hosts.

Lastly, the ability of snakes to act as reservoir hosts of group A arboviruses has been thoroughly demonstrated experimentally (18, 19). Our demonstration of the susceptibility of cultured cells of several reptilian species to infection by rabies virus suggest that investigation of a possible reservoir role for reptiles in maintaining rabies infection in nature may be justified. While early studies [reviewed by Babes (20)] revealed that poikilothermic vertebrates were apparently refractory to clinically evident rabies infection, the possibility of asymptomatic or "carrier" state infection has not been studied by modern methods.

Summary. The capacity of nine poikilothermic vertebrate cell lines, of fish, amphibian, and reptilian origin, to support replication of strain CVS rabies virus was determined. Two fish cell lines and two amphibian cell lines supported little or no rabies virus infection, while five reptilian cell lines tested varied from essentially unsusceptible (iguana cell line IgH2) to a high susceptibility (viper cell line VSW) comparable to that of mammalian cells. Rabies virus serially propagated in VSW cells became virtually apathogenic for adult mice by passage level 81, yet remained highly immunogenic. Rabies virus serially propagated in the gecko cell line GL1 became highly attenuated for adult mice by passage level 40, but also expressed reduced immunogenicity. In two separate experiments, virus passaged in GL1 cells also acquired a small-plaque character in early passage. Ibadan shrew virus and Lagos bat virus, viruses antigenically related to rabies virus, both replicated efficiently in cell line VSW, but only the Lagos virus could be serially propagated in GL1 cell culture.

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