When similar cultures are infected with both SV40 and adenovirus 12, after 72 hours 75% of the cells contain adenovirus particles and there is an increase in virus by titration. A similar enhancement of the growth of adenovirus 5 in AGMK cultures by SV40 has been observed. The development of progressive adenovirus-type cytopathic changes without demonstrable adenovirus particles in cells of AGMK cultures infected with adenoviruses alone suggests the possibility of some type of incomplete virus formation which is able to go to completion in the presence of SV40.

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Ability of a Fish Cell Line to Support the Growth of Mammalian Viruses. (29198)

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Venezuelan and Eastern equine encephalitis (VEE and EEE) viruses have been propagated in a wide variety of tissue cultures(1, 2,3). Most of these were derived from mammalian or avian sources, although Soret and Sanders(4) reported the growth of EEE virus in aquatic embryonic hosts maintained *in vitro*. Wolf and Quimby(5) recently established a continuous cell culture from tissues isolated from rainbow trout gonads (RTG). RTG cells are markedly different physiologically from mammalian or avian cells in that they (1) are incapable of growing above 25°C, (2) grow optimally at 22°C, and (3) are still capable of proliferating or maintaining themselves at temperatures as low as 4°C. This report deals with the growth and behavior of VEE and EEE viruses in the RTG cell line as compared with that in chick fibroblasts (CF) or L cells under the same cultural conditions.

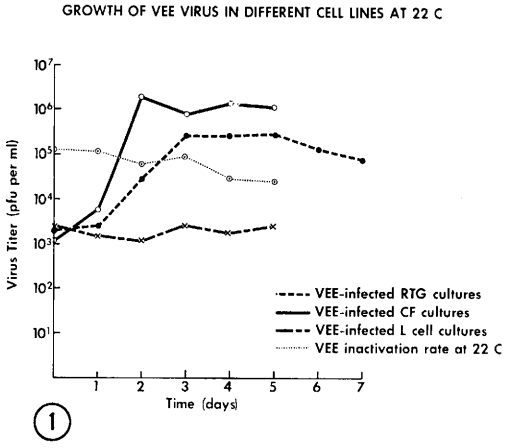
Materials and methods. Virus. The Trinidad strain of VEE virus(6) and the Louisiana strain of EEE virus(7) were used in these investigations. The virus seeds were prepared from infected chick embryos homogenized as a 10% suspension in heart infusion broth.

Tissue culture. The RTG cell cultures, obtained through the courtesy of Dr. K. Wolf, were grown at 22°C in Eagle's medium supplemented with 10% fetal calf serum. The CF cell cultures were prepared from 10-day-old chick embryos by the trypsinization method of McClain and Hackett(8). The CF and L cell monolayers were propagated at 37°C in the same medium as the RTG cells.

Inoculation of culture. The cell cultures, grown in 4-ounce prescription bottles, were infected with varying doses of VEE or EEE virus that had been diluted in growth medium. Other diluents such as phosphate-buffered saline were toxic to the RTG cultures. After inoculation with virus and an adsorption period of 15 minutes at 22°C, the cultures were washed 3 times, fed with growth medium, and incubated at 22°C unless otherwise specified. Samples were collected after 2 hours of incubation and daily thereafter.

Staining. RTG cell monolayers on 11 × 22 mm coverslips were infected with a virus multiplicity of approximately 30 plaque-forming-units (pfu) per cell. The representative cultures were removed at daily intervals, fixed in absolute methanol, and stained for 20 minutes in a 1:10 dilution of Giemsa.

Virus assay. All samples were assayed on CF cells by the suspended plaque technique described by Brown and Officer(9), with the following modification: An inoculum of 0.05



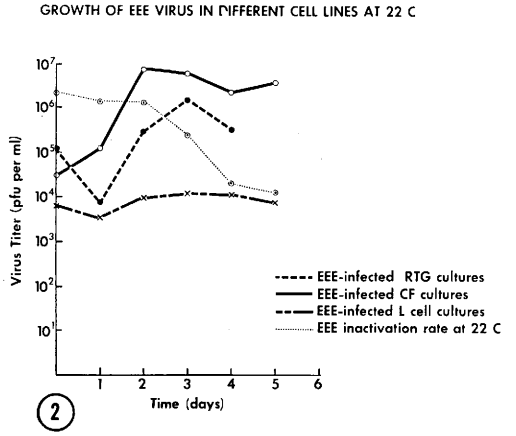


FIG. 1. Growth of VEE virus in different cell lines at 22°C.

FIG. 2. Growth of EEE virus in different cell lines at 22°C.

ml was spread on the surface of the suspended cells with a glass spreader. Plaques were counted on the second day of incubation at 37°C.

Results. Growth curves of VEE and EEE viruses. Growth curves of VEE and EEE viruses in the 3 different cell cultures after infection with a virus multiplicity of 50:1 are illustrated in Fig. 1 and 2. In Fig. 1 it can be seen that the VEE virus titer in RTG cultures at 48 hours showed a slight rise above the initial titer of 10^3 pfu/ml and then increased to a maximum at 72 hours, at which time there was more than a 100-fold increase. The titer remained constant at 5 $\times$ 10^5 pfu/ml for the next 48 hours, then started to decline. After 2 weeks of incubation the titer had declined to 10^3 pfu/ml. This titer persisted until termination of the experiment at 6 weeks. A very slight cytopathogenic effect (cpe) was evident in the RTG cultures during this period. The titer of VEE virus in CF cultures at the same temperature was about a log higher than that in the RTG cultures; moveover, CF cultures showed a pronounced cpe by 96 hours. No appreciable growth was obtained with VEE virus in L cell cultures at 22°C. RTG cultures infected with EEE virus also showed an increase in titer but the amount of growth was less than that obtained with VEE virus in RTG cultures (Fig. 2). The cultures infected with EEE virus also differed from those infected with VEE virus by showing an intense cpe after 2 days of incubation; by 96 and 144 hours the cultures were completely destroyed. The CF cultures infected with EEE virus also responded with higher titers and a cpe was evident after 96 hours of incubation. No measurable growth of EEE virus was obtained in the L cells incubated at 22°C.

Rates of adsorption at 4°C and penetration at 22°C of VEE virus were examined in replicate RTG cell cultures. The cell cultures were infected and incubated at 4°C. At different intervals of time, the cultures were removed and washed 3 times to remove most of the residual virus. Some of the cultures were then titrated immediately as infectious centers to determine the number of cells infected, others were incubated at 22°C to determine rate of penetration. VEE antiserum was used to neutralize adsorbed virus that had not yet penetrated the cells in the latter cultures. The results, summarized in Table I show that maximum adsorption occurs almost immediately and does not increase significantly upon further incubation. Rate of virus penetration at 22°C increased 100-fold during the 2-hour period.

The response of RTG cultures to different doses of EEE and VEE viruses is shown in Table II. The infecting doses ranged from 10^1 to 10^7 pfu per culture. The results indicated that the maximum titer obtained was related to, but not directly proportional to, the initial dose. RTG cultures infected with

TABLE I. Rate of VEE Virus Adsorption and Penetration in RTG Cultures.

Adsorption time (min at 4°C)	Incubation time (min at 22°C)	Time with anti-VEE serum (min at 22°C)	Infectious centers (plaque-forming units)
1	0	0	2×10^3
30	0	0	1.2×10^3
60	0	0	4.6×10^3
30	0	30	5.4×10^1
30	30	30	1×10^2
30	60	30	2×10^3
30	120	30	9.6×10^3

After each interval of adsorption and interval with antiserum, the cultures were washed 3 times with growth medium.

TABLE II. Growth of VEE and EEE Viruses in RTG Cultures as a Function of Inoculum Size.

Virus	Inoculum (pfu)	Incubation (days) at 22°C					
		0	1	2	3	4	5
VEE	$10^{7.0}$	4.2*	5.5	7.1	7.2	7.9	7.7
	$10^{5.0}$	4.1	5.2	7.3	6.9	6.7	7.4
	$10^{3.0}$	3.1	3.2	6.6	6.4	6.6	6.2
	$10^{1.0}$	<1.0	<2.0	<2.0	<2.0	<2.0	<2.0
EEE	$10^{7.0}$	4.3	<4.0	4.8†	<4.0	<4.0	
	$10^{5.0}$	3.3	5.1	7.4	7.6	7.3	6.8
	$10^{3.0}$	2.3	4.8	6.9	7.0	6.8	6.3
	$10^{1.0}$	$<10^1$	$<10^2$	6.3	6.5	6.8	5.8

* Plaque-forming units per ml expressed as the base $\log_{10}$.
† Complete cpe.

the highest multiplicities of VEE virus also yielded the highest peak titer. When the inoculum was reduced by more than 10,000-fold the resulting peak titer was only one log lower. Virus growth was not demonstrable in cultures infected with only 10 pfu of VEE virus. The RTG cultures infected with EEE virus responded somewhat differently from those infected with VEE virus. The highest inoculum of EEE virus appeared to be toxic for RTG cell cultures, since they showed a maximum cpe at 48 hours with only a slight rise in virus titer. When cultures were infected with a smaller inoculum of EEE virus, much higher titers were obtained than with the highest inoculum. As with VEE virus, the peak titers of EEE virus were higher in those cultures receiving the higher virus doses provided that the dose was not sufficiently high to be toxic to the cells. The cpe in RTG cultures was always more pronounced with EEE than with VEE virus.

To determine whether different temperatures of incubation would influence the rate of virus synthesis or the peak virus titer obtained, infected cultures were placed on a temperature gradient plate(10) that ranged from 20°C to 30°C. The results are presented in Table III. All RTG cultures incubated above 26°C, including uninfected controls, showed a pronounced cpe by 24 hours and were completely destroyed by 48 hours. No virus growth was demonstrable in these cultures. At 25°C the peak titer was slightly higher than those obtained at lower temperatures and it occurred at 24 hours rather than at 48 hours.

Uninfected and infected RTG cultures were fixed and stained at daily intervals, then examined for any morphological alteration of the cells. Uninfected RTG cultures remained quite vacuolated for 24 to 48 hours following transfer. These vacuoles usually appeared empty but on occasion contained basophilic material (Fig. 3). Later observations showed that any abnormal stress or cultural conditions produced the same effect. RTG cultures infected with VEE (Fig. 4) remained highly vacuolated but otherwise did not differ materially from uninfected cultures. The frequencies of mitotic figures in cultures infected with VEE virus and in uninfected cul-

TABLE III. Growth of VEE and EEE Viruses in RTG and CF Cell Cultures at Different Temperatures.

Virus strain	Cell strain	Temp (°C)	Incubation (days) 0	1	2	3	4	5
VEE	RTG	21	4.3*	4.7	5.9	6.0	6.0	4.9
		25	4.3	6.6	6.5	6.7	6.3	5.0
		30	4.2	4.6	<2.0	—	—	—
VEE	CF	21	3.3	5.7	6.3	7.0	—	—
		25	4.3	6.6	6.8	7.2	—	—
		30	4.5	6.3	7.0	—	—	—
		37	3.3	6.3	8.9	9.7	—	—
EEE	RTG	21	5.2	5.5	6.0	5.4	4.9	—
		25	5.8	6.3	6.2	6.2	5.7	—
		30	5.6	4.3	<2.0	—	—	—
EEE	CF	21	5.9	6.1	6.2	6.3	—	—
		25	5.7	7.0	6.8	6.8	—	—
		30	5.8	7.2	7.3	—	—	—
		37	4.9	7.4	9.2	9.0	—	—

* Plaque-forming units per ml expressed as the base $\log_{10}$.

tures were the same; therefore, infection with VEE virus did not alter the overall rate of cell proliferation. Cultures infected with EEE virus did show a progressive cpe in which the nucleus showed the first manifestation of infection. The nucleus first became wrinkled, then the chromatin began to condense into clumps (Fig. 5). The entire nucleus then began to disintegrate and disappeared from the cell. At this stage the cytoplasm lost its basophilic properties when stained with Giemsa, indicating a loss of cytoplasmic RNA.

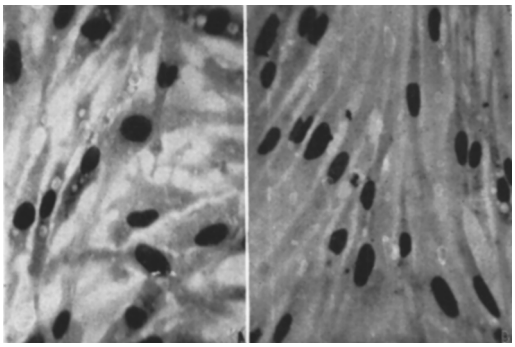


FIG. 3. Uninfected RTG cell cultures, Giemsa stained (× 172). A. 48-hr culture showing vacuolization. B. 96-hr culture. Cells appear normal.

Discussion. It is apparent from results reported here and elsewhere that the 2 arboviruses, VEE and EEE, are capable of infecting and proliferating in a variety of cell cultures derived from totally unrelated species, *i.e.*, mammalian, avian, and piscine. This suggests that these viruses, unlike cer-

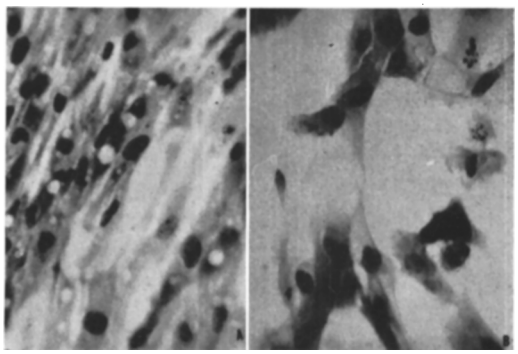


FIG. 4. VEE- and EEE-infected RTG cultures at 96 hr, Giemsa stained (× 172). A. VEE-infected RTG cultures. B. EEE-infected RTG cultures.

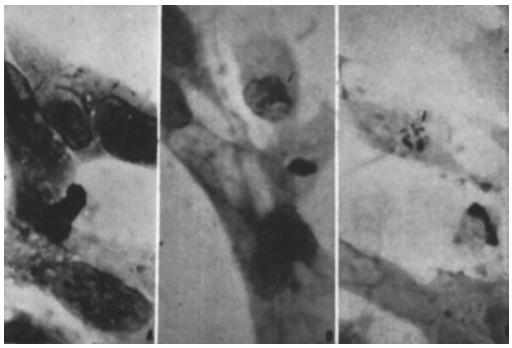


FIG. 5. EEE-infected RTG cell cultures showing manifestation of CPE in nucleus. Giemsa stained (× 430). A. Nuclear folding. B. Loss of basophilia and condensation of nuclear chromatin. C. Nuclear fragmentation.

tain human picornaviruses, *e.g.*, polio(11), do not require species-specific receptor sites to adsorb and penetrate cells.

Evidence is presented that the optimal proliferation of these viruses appeared to be directly proportional to the rate of metabolic activity of the cell. VEE(1) and EEE(3) viruses are both capable of growth in L cells at the optimal growth temperature for the cells (37°C). However, when L cells are infected and then incubated at temperatures below 25°C, they are incapable of supporting virus propagation.

The metabolic activities of CF cell cultures are not as restricted by different incubation temperatures as those of L cell or RTG cell cultures. Consequently, these cultures do support the growth of VEE and EEE viruses over a wide temperature range.

The RTG cell cultures made it possible to distinguish between these two species of virus. RTG cells were much more susceptible to the cytopathogenicity of EEE virus than VEE virus. Because of the lack of cytopathogenicity after infection with VEE, it was possible to obtain a persistent infection with this virus in RTG cultures in a manner similar to that obtained in L cells at 37°C(12).

Additional evidence that RTG cell cultures could be used to distinguish between the two virus species was obtained by examining stained preparations of infected cultures. VEE virus infection did not produce any observable microscopic change in the RTG culture other than prolonged vacuolization. EEE virus infection, on the other hand, caused a progressive characteristic cpe in RTG cells. At higher magnification a definite pattern of cytopathogenicity, with distinct nuclear changes as the first manifestation, was easily observed in RTG cultures infected with EEE virus.

Summary. A fish cell line derived from rainbow trout gonads (RTG) has been shown to support the proliferation of 2 arboviruses, Venezuelan equine encephalitis (VEE) virus and Eastern equine encephalitis (EEE) virus, at 22°C. EEE virus was more cytopathogenic for RTG cultures than VEE virus, thus making it feasible to distinguish between the two. A persistent infection was obtained with VEE virus for as long as cultures were carried, but not with EEE virus. Both viruses multiplied in chick fibroblast (CF) cells at 22°C to slightly higher titers than in the fish cell line and both viruses caused a cytopathogenic effect in CF cells. L cells failed to support the growth of either virus at 22°C.

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