Studies of Replication of Four Selected Viruses in Two Cell Lines Derived from Salmonid Fish[1,2] (34976)

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Cell lines derived from embryos of four species of salmonid fish have been established in this laboratory. Certain of these lines and the methods used to establish them have been described previously (1, 2). In characterizing autonomous lines of animal cells, the spectrum of virus susceptibility is always of interest. These salmonid fish cell lines may be considered to have special interest in this respect because of their low optimum temperature range, 18–23°. The experiments reported herein represent the beginning of a study intended to define the viral spectrum of these cells. The established cell lines selected for this work were STE-137 from steelhead trout embryos (*Salmo gairdneri*), and CHSE-114 from chinook salmon embryos (*Oncorhynchus tshawytscha*). Four viral agents were selected for study, two of which were isolated from salmonid fish, the sockeye salmon virus (Oregon strain), (3) designated OSV and the Sacramento River chinook virus, or SRCV. Two viruses of warm-blooded vertebrates were included, the western equine encephalitis virus or WEE, a representative of the arbovirus group, and the agent of Newcastle disease, or NDV, a myxovirus.

Materials and Methods. Cell cultures. Four lines of cells derived from the embryonic tissues of salmonid fish species were employed in the experiments reported. Cell line No. STE-137, had been carried through 62 cultural passages at 2- to 3-week intervals when this work was started. Cell lines CHSE-114 and CHSE-214 from chinook salmon embryos, were in their 66th and 53rd culture passages, respectively. Cell line SSE-5, from sockeye salmon embryos (*Oncorhynchus nerka*) had been through approximately 25 culture passages, and is not yet considered an "established" line. STE-137 and CHSE-114 are heteroploid, while no chromosome analysis has been made of the other two lines.

All cell lines were maintained as monolayer cultures in Eagle's minimum essential medium (MEM) (4) supplemented with 20% agamma calf serum (Hyland Laboratories). The complete medium also contained 100 units of penicillin, 100 μg of streptomycin, and 25 units of mycostatin/ml. The details of methods employed for maintenance of stock cultures and preparation of experimental cultures have been described (10). The cell lines were tested for mycoplasma contamination by both cultural and chemical methods, but none was detected.

Viruses. OSV. The isolation of this virus and its maintenance in cultures of sockeye salmon embryonic cells have been reported (3). The culture fluid used as stock virus was stored at −60°, and the virus concentration was $10^{5.6}$ $TCID_{50}$/ml.[3]

SRCV. This virus was originally obtained from tissues of young chinook salmon during the 1965 epizootic at the Coleman National Fish Hatchery in California. It was received from Dr. W. H. Wingfield of the California Department of Fish and Game Laboratory in Sacramento, as frozen tissue culture fluids from infected cultures of chinook salmon embryonic cells. Stock virus was prepared by inoculating cultures of CHSE-214 cells with approximately a 1:50 dilution of the frozen culture fluids and incubating at 10° for 5 days. The culture fluid was centrifuged at low speed, dispensed in small volumes, and stored at −60°. The virus concentration was $10^{4.8}$ $TCID_{50}$/ml.[3]

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[2] Technical paper No. 2807, Oregon Agricultural Experiment Station.

[3] Fifty per cent infectious virus dose for cell cultures.

WEE. The virus used was the California strain, isolated from horse brain in 1931 by K. F. Meyer, C. M. Haring, and B. F. Howitt (6). It has been passed an unknown number of times in mouse brain, guinea pig brain, and chick embryos. Stock virus for these experiments was prepared from minced infected chick embryo, which had been stored at —60°. A 1:10 dilution was prepared in Eagle's MEM by triturating the tissue in a sterile mortar. After low-speed centrifugation 10-day chick embroys were inoculated in the allantoic cavity with 0.1 ml of the diluted virus. After 48 hr incubation at 35° the embroys were aseptically removed, the legs and beak discarded, and the remaining tissue minced with scalpels. This pulp was dispensed in small volumes and stored at —60°. The virus concentration was $10^{6.4}$ $CEID_{50}/g$.[4]

NDV. This agent, a Montana strain, was obtained from the Department of Veterinary Medicine, Oregon State University as frozen allantoic fluid, which had been held at —60°. Stock virus was prepared by inoculating 10-day chick embryos in the allantoic sac with 0.1 ml of a 1:10 dilution of the thawed allantoic fluid. After 48 hr incubation at 35° allantoic fluid was collected aseptically from the inoculated eggs, centrifuged at low speed, dispensed in small volumes, and stored at —60°. The virus concentration was $10^{6.8}$ $CEID_{50}/ml$.[4]

Methods of titrating infectious virus. OSV. Virus concentration in fluids of experimental cultures was measured by the end point dilution method, employing monolayer cultures of SSE-5, cells growing in 75 × 12-mm Pyrex tubes as previously described (10).

SRCV. Fluids containing this virus were titrated by a method essentially the same as that used for OSV, except that monolayer cultures of CHSE-214 cells were employed, and these were incubated at 10° for 7 days, instead of 18°. They were then examined for cytopathic effects.

WEE. The virus was titrated in 9- to 11-day chick embryos. Ten-fold dilutions of the fluid to be titrated were prepared in complete medium. For each dilution five embryos were inoculated with 0.1 ml in the allantoic sac. The eggs were incubated at 35° for 72 hr. They were candled at 48 and 72 hr to observe death of the embryo. The 50% endpoint for infectivity was then estimated by the Reed and Muench method (7).

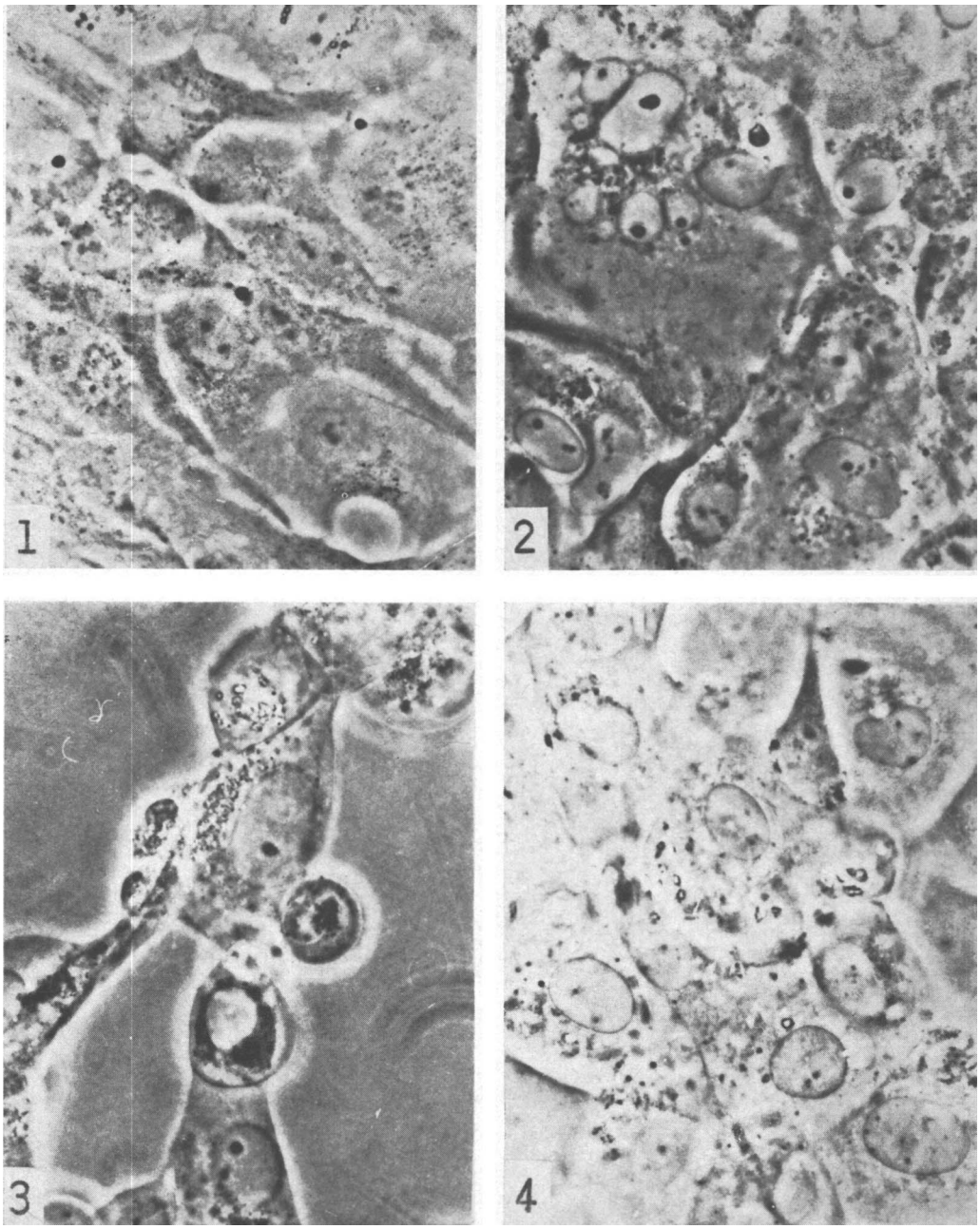
NDV. The strain of the virus used regularly produced death in 9- to 11-day chick embryos within 4 days after inoculation. The method of titrating virus infectivity was exactly the same as for WEE virus, except that

TABLE I. Replication and Serial Propagation of Sockeye Salmon Virus (OSV) in Salmonid Cell Lines at 18°.

		Log of $TCID_{50}$/0.5 ml of culture fluid				
		Single-passage experiments			Five serial passages	
Cell line	Days of incubation	1	2	3	First culture passage	Fifth culture passage
STE-137[a]	0	2.6	2.5	2.8	1.2	3.5
	5	6.2	6.2	5.6	>6.0	7.4
CHSE-114[b]	0	2.6	2.5	2.8	2.6	2.6
	5	3.8	3.4	2.8	3.8	5.2
SSE-5[c]	0	2.6	2.5	2.8	1.2	2.5
	5	6.8	6.3	6.2	>6.0	6.6

[a] Steelhead trout cell line.
[b] Chinook salmon cell line.
[c] Sockeye salmon cell line.

[4] Fifty per cent infectious virus dose for 10-day chick embryos.



FIGS. 1–4 are photographs of cell cultures in Rose chambers taken by phase contrast microscopy. 490×.

FIG. 1. Uninfected SSE-5 cells (sockeye salmon) of approximately the same age as cells in Fig. 2.

FIG. 2. SSE-5 cells 3 days after inoculation with OSV. Note thickening of nuclear membranes and increased optical density of nucleoli.

FIG. 3. SSE-5 cells 6 days after inoculation with OSV. Many cells rounding up and sloughing from glass surface.

FIG. 4. STE-137 cells (steelhead trout) 2 days after inoculation with SRCV. Note similarity to Fig. 2.

eggs were incubated for 4 days and examined for embryo deaths after 72 and 96 hr.

Results. OSV. In experiments with OSV, cell cultures were inoculated from stock virus which had been stored at —60°. After thawing, it was diluted in complete medium to a concentration of approximately $10^{2.5}$ $TCID_{50}$/0.5 ml. The old medium was removed from two Leighton tube cultures of the cell line to be inoculated and replaced with 1 ml of the diluted virus. Inoculated tubes were then incubated at 18° for 5 days. Infectious virus was titrated in the original inoculum and again in the pooled culture fluids at the end of the incubation period. This was done by the end-point dilution method, using small tube cultures of SSE-5 cells, as described (10).

Results obtained with this virus are shown in Table I. The data show that in the single-passage experiments with cell line STE-137 the virus concentration in the culture fluids increased by 3 or more log units. For comparison, parallel experiments were carried out in cultures of SSE-5 cells, derived from embryonic tissues of the natural host for this virus. The yield of new virus in these cultures was not significantly different from that in the STE-137 cells. The virus was then carried through five serial passages in each of these cell lines. The yield of virus in the fifth-series cultures was at least as high in the STE-137 cells as in SSE-5. Thus the steelhead trout cells supported replication of this virus as well as cells from the natural host species.

Along with these studies of virus propagation, the cytopathic effect of the agent in both of the above cell lines was recorded. For this purpose cell cultures were prepared in Rose chambers (8) and after a monolayer had formed the culture fluid was replaced with complete medium containing virus in a concentration of $10^{2.5}$ $TCID_{50}$/0.5 ml. The chambers were incubated at 18° and examined daily by phase-contrast microscopy. The changes produced in cells by infection with this virus were quite characteristic. They first became evident after 48 hr, when the nuclear membranes appeared distinctly thickened and the optical density of the nucleoli was markedly increased (Fig. 2). By the sixth day of infection many cells had become spherical in shape and many others were detached from the glass (Fig. 3). The same cytopathic changes were noted in both STE-137 and SSE-5 cells.

The behavior of the OSV virus in the chinook salmon cell line (CHSE-114) was somewhat different. In the single-passage experiments, infectious virus increased by only 1 log in two cases, and not at all in a third. No cytopathic changes could be detected in these cell cultures. The virus was able to replicate to only a very limited extent in this cell line after the primary exposure. It was, however, possible to transmit the agent serially in these cells, and during the course of five serial passages the virulence of the virus for these cells appeared to increase. This is indicated by the fact that in the fifth culture of the series the increase in infectious virus was greater than in single-passage experiments, and by the appearance of the typical cytopathic changes in the cells. The yield of progeny virus was still below that produced in the other two cell lines, but might well have been increased still more by continued transfer in the CHSE-114 cells.

SRCV. This virus was studied in experiments with cell lines STE-137 and CHSE-114 which were closely similar to those employed with OSV. Leighton tube and Rose chamber cultures were inoculated with stock virus in a concentration of $10^{3.5}$ $TCID_{50}$/0.5 ml and incubated at 10°. Infectious virus was titrated in fluids of tube cultures on the day of inoculation and again on the tenth day of incubation.

The data in Table II indicate that replication of this virus occurred in single-passage experiments with both cell lines. The agent was readily transmitted through a series of five passages in both lines. It may be noted that in most experiments the yield of infectious virus from CHSE-114 cells was slightly lower than from STE-137. The cytopathic effects produced by SRCV in both lines of cells were closely similar to those seen in cells infected by OSV (Fig. 4).

WEE. Experiments carried out with the WEE virus were very similar to those described in the study of the two fish viruses.

TABLE II. Replication and Serial Propagation of Sacramento River Chinook Virus (SRCV) in Salmonid Cell Lines at 10°.

Cell line	Days of incubation	Log of $TCID_{50}$/0.5 ml of culture fluid				
		Single-passage experiments			Five serial passages	
		1	2	3	First culture passage	Fifth culture passage
STE-137[a]	0	3.2	4.5	3.8	3.8	3.4
	10	6.2	5.5	7.3	7.3	7.2
CHSE-114[b]	0	3.2	4.5	3.8	3.8	4.3
	10	4.8	5.5	6.5	6.5	6.5

[a] Steelhead trout cell line.
[b] Chinook salmon cell line.

Early trials indicated that cultures of the salmonid cells degenerated and sloughed off the glass surface when exposed to temperatures above 26°, and no virus replication could be detected in infected cultures under these conditions.

The results of experiments with the WEE virus in STE-137 and CHSE-114 cell cultures incubated at 23 and at 26° are presented in Table III. In the single-passage experiments there was no significant virus replication in STE-137 cultures at 23° in the 7-day period, while at 26° infectious virus increased by almost 4 log units. In CHSE-114 cultures at 23° there was an increase of about 1.6 logs, while at 26° the increase was over 6 logs. The critical influence of incubation temperature and the near optimal value of 26° for replication of the virus are thus clearly indicated. For comparison, the virus yield in cultures of chick fibroblasts, at 35° was measured and a 4-log increase found. The virus was carried through five serial passages in cultures of both fish cell lines, with the results shown in Table III. The virus yield in the fifth culture of the series represented an increase of over 3 log units in both cases. There seems little doubt that the virus could be propagated indefinitely in these cultures.

TABLE III. Replication and Serial Propagation of Western Equine Encephalitis Virus in Salmonid Cell Lines.

Cell lines	Days of incubation	Log of $CEID_{50}$/0.1 ml of culture fluid				
		Single-passage experiments			Five serial passages at 26°	
		23°	26°	35°	First culture passage	Fifth culture passage
STE-137[a]	0	1.6	1.4	—	3.3	1.5
	2	1.6	4.7	—	—	—
	5	—	—	—	5.8	4.9
	7	1.8	5.3	—	—	—
CHSE-114[b]	0	1.6	1.4	—	3.3	2.5
	2	2.0	4.6	—	—	—
	5	—	—	—	6.0	5.8
	7	3.2	7.8	—	—	—
Chick fibroblasts	0	—	—	1.2	—	—
	3	—	—	5.2	—	—

[a] Steelhead trout cell line.
[b] Chinook salmon cell line.

Infection with the WEE virus produced pronounced cytopathic effects in both lines of cells. By the third day of infection some of the cells had begun to assume a round and granular appearance and others had become detached from the glass. These changes were more pronounced on the fifth day. The most obvious cellular alterations consisted of shrinkage of the nuclei and condensation and vacuolization of the cytoplasm. These changes are not unique to cells infected with the WEE virus, and are very similar to the cytopatic effects of this agent in mammalian and avian cells (9). By the seventh day of infection, nearly all cells in an infected monolayer had sloughed off the glass.

NDV. The ability of NDV, a myxovirus, to replicate in the salmonid cell lines was also explored. Leighton-tube cultures of STE-137 and CHSE-114 cells were inoculated with virus diluted to about $10^{3.5}$ $CEID_{50}$/0.1 ml. Some tubes were incubated at 23° and others at 26°. Infectious virus in culture fluids was titrated in 9- to 11-day chick embryos on the day of inoculation and again after 2 and 7 days incubation. No increase in virus titer was ever found, and a decrease was usually noted on the seventh day. In HeLa cell cultures similarly inoculated and incubated at 35°, infectious virus increased by 3.6 log units in 48 hr. A series of five blind passages of the virus was carried out in each of the salmonid cell lines, but no significant amounts of activity were detected in fluids of the fifth series of cultures. All experiments supported the conclusion that NDV was unable to replicate in either cell line.

Discussion. It is of interest to compare the species specificity shown by the OSV in fingerling salmonids with its behavior in cell cultures derived from salmonid species. As reported earlier, when fingerling sockeye, chinook and coho salmon, and rainbow trout were exposed to the virus in their water supply, most of the sockeye salmon developed fatal infection, while most of the other groups remained symptom-free (10). However, the STE-137 cells, derived from steelhead trout[5] embryos proved to be as susceptible to infection as SSE-5 cells, derived from sockeye salmon, the natural host. The chinook salmon cell line, CHSE-114, supported limited replication on primary exposure to the virus, and greatly increased yields after adaptation had occurred. Various factors could have contributed to the difference in behavior of the agent in fingerlings and in cell cultures. Some of these may be factors which are a part of the defense mechanisms of the intact salmonid host. Others may be unidentified changes which have taken place in cell lines transferred repeatedly in the laboratory over an extended period of time; for example, development of the heteroploid state. The possible role of such a change is suggested by the results of one experiment conducted in this laboratory in which a cell line derived from chinook salmon embryos, and in its first transfer from the primary culture, failed to support replication of OSV.

The cytopathic effects produced by OSV and SRCV were most distinct in the cell nuclei, which strongly suggests that at least part of the replicative process takes place in that portion of the host cell. The fact that the cellular changes accompanying infection with these two agents are essentially identical suggests that they may be closely related viruses. Possible immunological relationships are currently being investigated. Studies by electron microscopy are also needed.

It is also of some interest that the WEE virus could replicate and presumably be propagated indefinitely in these salmonid cell lines, with a resulting virus yield at least as high as that found in a culture of chick fibroblasts. The virus of eastern equine encephalitis has been propagated in embryos of *Gambusia*, a viviparous fish (11). Both the eastern and the Venezuelan equine encephalitis viruses have been shown to replicate in cultures of cells derived from gonad tissue of rainbow trout at 22°, though the virus yield was lower than in chick fibroblasts (12). The WEE virus, although similar to the eastern and Venezuelan agents in many respects, is immunologically unrelated to them. Its ability to replicate in these cell lines derived from a poikilothermic animal at

[5] The steelhead and rainbow trout are the same species of animal; steelhead are the sea run form of rainbow trout.

a temperature well below that of its natural hosts suggests the possibility that continuous serial propagation in these cells over an extended period might well result in the development of a strain possessing a low degree of virulence for the natural hosts. It also suggests the possibility that this virus might produce infection in poikilothermic animals in nature in climates where the body temperature does not fall below 24 or 25°, and assuming the presence of an arthropod vector capable of transmitting infection. WEE has been found to survive in experimentally infected garter snakes after hibernation over the winter under simulated natural conditions (13, 14).

Summary. Two established cell lines, derived from the embryonic tissues of chinook salmon and steelhead trout, respectively, have been examined for their ability to support the replication of two viruses of salmonid fish, and two viruses of warm-blooded vertebrates. The sockeye salmon virus (Oregon strain) produced large yields of progeny virus upon primary inoculation in the steelhead trout cells, and was readily propagated through five serial transfers. Infection was accompanied by a characteristic cytopathic effect on the cells. In the chinook salmon cells, replication of the virus was limited upon primary inoculation, but was greatly enhanced after a series of five culture passages. The Sacramento River chinook virus was also found to replicate in both of these cell lines upon primary inoculation, and the agent was transmitted through five serial passages. The cytopathic effect produced by this virus appeared essentially identical to that of the sockeye salmon virus. It was found that both salmonid cell lines would support replication of the western equine encephalitis virus very adequately at 26°. At 23° replication of the virus was very limited in the chinook salmon cells and none could be detected in the steelhead cells at the end of a 7-day incubation period. The virus was propagated through five serial transfers in both cell lines, with the production of a marked cytopathic effect. The virus of Newcastle disease failed to replicate in either cell line.

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