

Two Cell Lines from White Sturgeon

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Abstract.—Cell lines were established from spleen and heart tissues of a juvenile white sturgeon *Acipenser transmontanus* and designated WSS-2 and WSH-1, respectively. Isoenzyme analyses of both lines confirmed their host of origin. Both lines were hypotetraploid and possessed micro- and macrochromosomes. The modal chromosome number for the WSS-2 line was 219; the WSH-1 line had a bimodal distribution with modes of 237 and 243. The effects of temperature on growth and susceptibility to selected viral pathogens were examined for the WSS-2 line. Cell replication occurred at 15–30°C, optimum temperature was 25°C. Cell survival was observed after 11 d at 10°C but not at 35°C. The WSS-2 line was refractory to infections with channel catfish virus (CCV) and infectious pancreatic necrosis virus (IPNV) but susceptible to infectious hematopoietic necrosis virus (IHNV) and white sturgeon iridovirus (WSIV). The need for cell lines from the host of origin in studies of viral pathogens of fish was demonstrated by the ability of the WSS-2 line to support replication of WSIV.

Cell lines have been established from numerous species of fish, and many are routinely used for the diagnosis and study of piscine viruses. Wolf and Mann (1980) listed 75 lines from 35 species of fish. However, only two cell lines of sturgeon origin exist: the SH line, from cardiac tissue of the Atlantic sturgeon *Acipenser oxyrinchus* (Li et al. 1985), and the SF line from fin tissues of the hybrid of beluga *Huso huso* and sterlet *A. ruthenus* (N. Okamoto, Tokyo University of Fisheries, personal communication).

The need for host-specific cell lines for the white sturgeon *Acipenser transmontanus* became evident as losses thought to be of viral etiology occurred among alevin and juvenile white sturgeons reared in research and commercial facilities in California. Three viruses—an adenovirus, an iridovirus, and a herpesvirus—have now been detected among farmed populations of white sturgeon in northern California (Hedrick et al. 1985, 1990; unpublished data). All three viruses have been associated with above-normal losses of juvenile white sturgeons, yet none can be isolated

by means of standard cell lines (Amos 1985) found in fish health laboratories. Of the three viruses, the white sturgeon iridovirus (WSIV) is considered the principal viral pathogen because of its association with annual mortalities among juvenile white sturgeons reared in several farms in northern California (Hedrick et al. 1990) and the Columbia River drainage in Oregon (unpublished data). The pathogenic nature of WSIV has recently been demonstrated by 100% mortality of juveniles following bath exposures to the virus grown in spleen cells from white sturgeon (our unpublished data).

White sturgeons are routinely moved from production facilities for use in the ornamental and food fish markets and for stocking public waters. The potential spread of viral agents has created a need to detect the viruses during fish health examinations by means that include the use of white sturgeon cell lines. The purpose of the present paper is to describe the establishment, morphology, and verification of host origin of two new cell lines from white sturgeons. The effects of temperature

on growth and susceptibility to selected fish viruses (including WSIV) of one of the lines are also described.

Methods

Establishment of cell lines.—Spleen and heart tissues were aseptically removed from a juvenile white sturgeon (45 g) and minced into 1-mm³ pieces in Hank's balanced salt solution (HBSS; Grand Island Biological Co. [GIBCO], McLean, Virginia). Tissue fragments were dispersed by treatment with 0.25% trypsin in HBSS for 30 min at 20°C with intermittent agitation. The cells were rinsed in HBSS, centrifuged at 1,500 × gravity (g) for 10 min at 4°C, and then resuspended to a concentration of 5 × 10⁵ cells/mL in minimal essential medium (MEM) with Earle's salts (GIBCO) containing 15% fetal bovine serum (FBS; Hyclone Laboratory Inc., Logan, Utah), 100 IU penicillin and 100 µg streptomycin per milliliter, and 2 mM L-glutamine (GIBCO). Five-milliliter cell suspensions from each tissue were placed into duplicate 25-cm² flasks (Corning Co., Corning, New York) and incubated at 25°C.

Monolayer cultures obtained after 21–30 d of incubation at 25°C were subcultured with a trypsin (0.025%) and EDTA (0.05%) solution. The cells were suspended in MEM containing 10% FBS (MEM-10) and incubated at 25°C. We named the heart cell line WSH-1 and the spleen line WSS-2.

The WSH-1 and WSS-2 lines, at subcultures 22 and 30, respectively, were tested for the presence of mycoplasma contamination with a commercially prepared medium, MYCOTRIM TC (Irving Scientific, Santa Ana, California), designed for this purpose.

Effect of temperature on growth.—To determine the effect of temperature on the growth of WSS-2 cells at subculture 25, replicate cell cultures were incubated at 10, 15, 20, 25, 30, and 35°C. A suspension containing approximately 5 × 10⁴ WSS-2 cells in MEM-10 (pH 7.6) with 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was plated into replicate 35-mm dishes with premarked grids. Replicate dishes (12) were incubated at each temperature, plus an additional two dishes at 20°C. At selected intervals, two dishes were withdrawn from each temperature and the medium was gently removed. The cells were then treated with 10% neutral buffered formalin for 1 h. The formalin was removed, and the cells were rinsed three times in 0.01 M phosphate-buffered saline (pH 7.6) and stained with 0.1% crystal violet to aid in counting. The mean number of cells

per square centimeter was determined by counting cells in premarked grids of duplicate dishes. The initial cell count (day 0) was determined by counting the mean number of cells attached after 2 h in two dishes at 20°C.

Isozyme analysis of cells.—To confirm the species of origin, WSH-1 and WSS-2 cells (subcultures 19 and 25, respectively) were compared by isozyme analyses with spleen and heart tissues removed directly from juvenile white sturgeons. Monolayers of approximately 10⁷ cells of each line were rinsed four times in MEM without serum (MEM-0). The cells were then scraped from the flask, suspended in 5 mL of MEM-0, and centrifuged at 1,500 × g for 10 min at 4°C. Fragments (1 mm³) from the heart and spleen of juvenile sturgeons were removed and washed four times in MEM-0 at 4°C for isozyme comparisons with cell lines. All samples were homogenized and applied to starch gels, and the proteins were separated by electrophoresis and stained as described by O'Brien et al. (1980). A panel of enzymes was used, and adenylate kinase (AK, enzyme number 2.7.4.3 of IUBNC 1984), glucose-6-phosphate isomerase (GPI, 5.3.1.9), and the peptidase glycyl leucine (GL, 3.4.11/.13) were found to be specific for identification of host gene loci.

Karyology.—The chromosomes of the WSS-2 and WSH-1 lines were counted at subcultures 24 and 12, respectively, with the techniques for chromosome spreading and enumeration described by Early (1975). One hundred chromosome spreads for each cell line were counted at 1,000 × magnification with an ocular grid.

Susceptibility to viruses.—The susceptibility of the WSS-2 line to infectious pancreatic necrosis virus (IPNV, strain VR-299; Wolf et al. 1960), infectious hematopoietic necrosis virus (IHNV, Metolius River strain; Mulcahy et al. 1980), and channel catfish virus (CCV; Wolf and Darlington 1971) was determined at subculture 29. The susceptibility of the WSS-2 subculture 55 to WSIV was also examined. Briefly, WSS-2 cells were grown to 90% confluency in 25-cm² flasks with MEM-5. The culture fluid was removed and duplicate flasks were inoculated with 10^{2.5} to 10^{4.6} 50% tissue culture infective doses (TCID₅₀) of each virus in 0.2 mL MEM-5. After a 30-min adsorption period, 5 mL of MEM-5 were added to each flask. The flasks were then incubated at a temperature appropriate for each virus (see below).

As positive cell controls, cultures of known susceptible lines were grown, inoculated, and incubated under the same conditions as those de-



FIGURE 1.—Micrographs of the two cell lines from white sturgeon: (A) the WSS-2 line derived from spleen; (B) the WSH-1 line from heart. Giemsa stain. Bars = 1 mm.

scribed for the WSS-2 line. The cyprinid cell line EPC (epithelioma papulosum cyprini; Fijan et al. 1983) and the chinook salmon embryo line CHSE-214 (American Type Culture Collection, ATCC CRL 1681; Lannan et al. 1984) were used for IHNV and IPNV, respectively. The channel catfish ovary (CCO) line, of ictalurid origin (Bowser and Plumb 1980), was used for CCV.

Immediately after addition of medium, a 0.1-mL aliquot was removed from each flask and the virus concentration was determined by TCID₅₀ assay on the known susceptible line. The concentrations of virus found in the medium of WSS-2 and known susceptible lines were again determined after 3 d at 20°C for IPNV, 7 d at 15°C for

IHNV, 3 d at 25°C for CCV, and 21 d at 20°C for WSIV. Differences between initial and final concentrations were then compared.

Results

Establishment of Cell Lines

Between 21 and 30 d after initiation, monolayer cultures were obtained from spleen and heart tissues. These cells grew readily throughout early passages and have continued without evidence of crisis periods to 55 and 43 subcultures for the WSS-2 and WSH-1 lines, respectively. An increasingly higher cell survival following dispersment with trypsin-EDTA was found with succes-

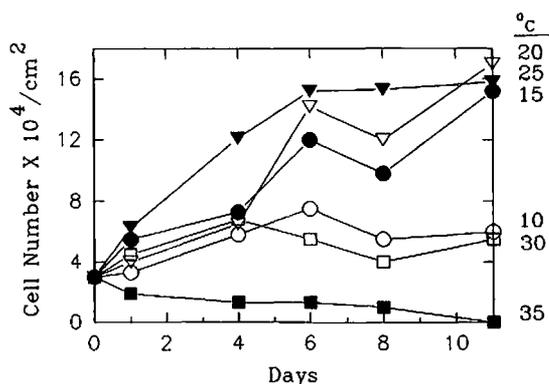


FIGURE 2.—Growth of the white sturgeon spleen cell line (WSS-2) at selected temperatures.

sive subcultures of both lines. The spleen line (WSS-2) was more epithelioid, whereas the heart line (WSH-1) appeared more fibroblastic. Cell populations in both lines were not homogeneous (Figure 1A, B). No mycoplasma contamination was detected in either cell line.

Effect of Temperature

The WSS-2 line grew well at 15, 20 and 25°C, but limited growth or survival was observed at 10 and 30°C (Figure 2). Cells incubated at 35°C had

TABLE 1.—Concentrations of infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), channel catfish virus (CCV), and white sturgeon iridovirus (WSIV) following inoculation of the WSS-2 sturgeon cell line.

Virus	Cell line	Concentration of virus (\log_{10} TCID50/mL) ^a		
		Initial titer	Final titer	Difference
IHNV ^b	EPC	4.6	8.3	3.7
	WSS-2		7.0	2.4
IPNV ^c	CHSE-214	3.5	9.0	5.5
	WSS-2		1.4	-2.1
CCV ^d	CCO	3.9	8.5	4.6
	WSS-2		3.7	-0.2
WSIV ^e	WSS-2	2.5	5.5	3.0

^a Average of duplicate titrations. TCID50 is the (tissue culture infective) dose of virus that induced a cytopathic effect in 50% of inoculated cultures.

^b Cyprinid (EPC) and sturgeon spleen (WSS-2) cells were incubated at 15°C for 7 d. The virus in the supernatant of both lines was determined by TCID50 analyses in EPC cells.

^c Chinook salmon cells (CHSE-214) and WSS-2 cells were incubated at 20°C for 3 d. The virus in the supernatant of both lines was determined by TCID50 analyses in CHSE-214 cells.

^d Channel catfish ovary (CCO) and WSS-2 cells were incubated at 25°C for 3 d. The virus in the supernatant of both lines was determined by TCID50 analyses in CCO cells.

^e WSS-2 cells were incubated at 20°C for 21 d. Virus in the supernatant was determined by TCID50 analyses on WSS-2 cells.

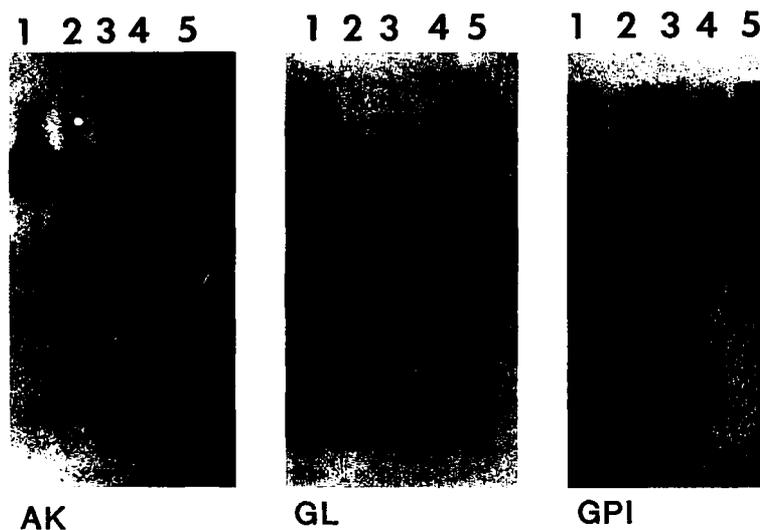


FIGURE 3.—Isoenzyme profiles for two white sturgeon cell lines. Adenylate kinase (AK): lane 1—WSS-2; 2 and 3—spleen; 4—WSH-1; 5—heart. Glycyl leucine peptidase (GL): 1—spleen; 2 and 3—WSS-2; 4—WSH-1; 5—heart. Glucose-6-phosphate isomerase (GPI): 1—spleen; 2 and 3—WSS-2; 4—WSH-1; 5—heart. Electrophoresis was from top to bottom.

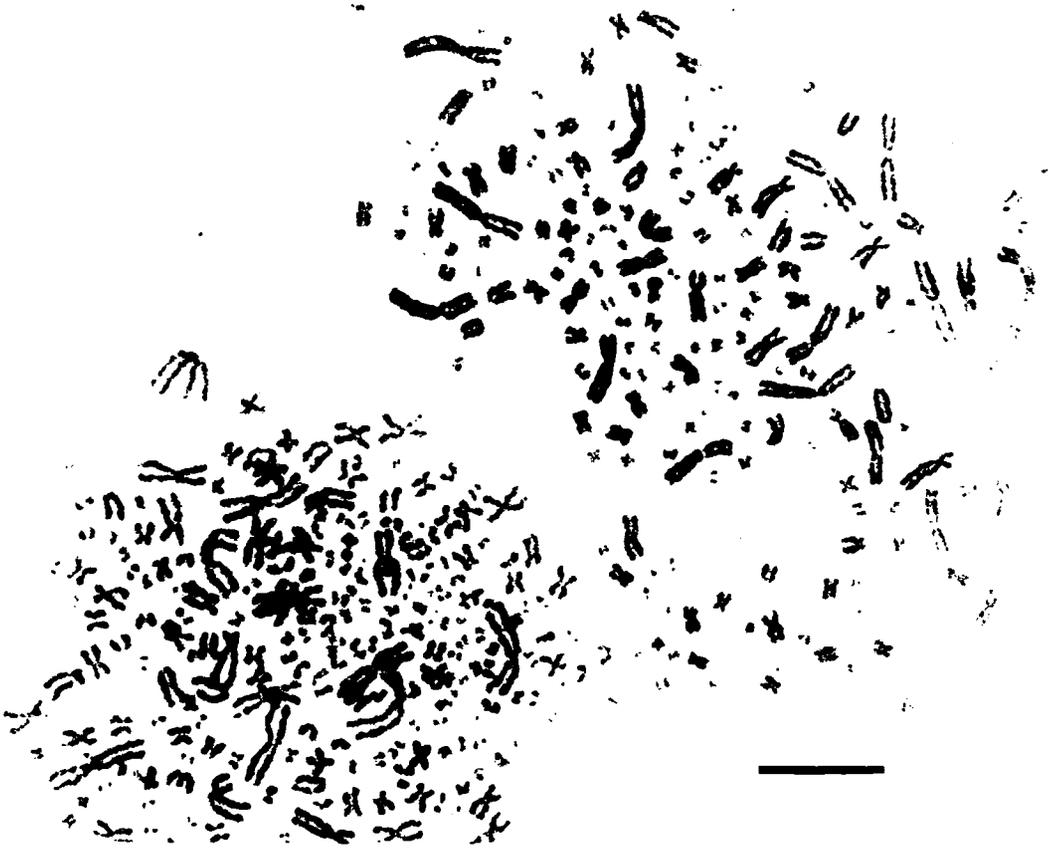


FIGURE 4.—Metaphase chromosomes from the white sturgeon cell line WSS-2. Giemsa stain. Bar = 200 μ m.

all died by day 11. Cell growth was most rapid at 25°C, but comparable cell densities were reached at 15 and 20°C by day 11 (Figure 2).

Isozyme Analyses

Electrophoretic comparisons of freshly removed heart and spleen tissues with the WSS-2 and WSH-1 cells implied similar loci for the three enzymes (Figure 3). Stains for GPI indicated the greatest similarity between the two cell lines and tissues from the host of origin (Figure 3). The GL and AK enzyme stains revealed similar banding patterns, although they were less distinct for the two cell lines than for the fresh tissues (Figure 3). One less locus was represented in the AK enzyme from both the WSS-2 and WSH-1 cells than in samples from fresh tissues.

Karyology

A wide distribution of chromosome numbers was found in the 100 cells examined for each line. The WSH-1 line had a bimodal distribution with modes of 237 and 243 chromosomes. The WSS-2 line showed a tighter grouping of chromosomes around a mode of 219. Both micro- and macrochromosomes were found in metaphase-arrested cells (Figure 4).

Susceptibility to Viruses

Neither cytopathic effects (CPE) nor an appreciable increase in virus titer was observed in WSS-2 cells after inoculation with IPNV or CCV (Table 1). However, typical IHNV CPE was found within 3 d of inoculation at 15°C. The final titer of IHNV from WSS-2 cells was less than that observed from

the EPC cell line. The three viruses not of sturgeon origin grew well in their respective control cells, reaching concentrations between 10^8 and 10^9 TCID₅₀/mL of cell culture supernatant (Table 1). The WSIV induced CPE after 14 d at 20°C and the yields of infectious virus were $10^{5.2}$ TCID₅₀/mL.

Discussion

The specificity of certain pathogenic viruses for cells from the same family or genus of fish has required development of numerous cell lines (Wolf 1988). The observation of viruses as causes of mortality among farmed populations of white sturgeon and the absence of host-specific cell lines for viral detection prompted us to develop cell lines for the detection and isolation of these viral agents.

The white sturgeon heart and spleen tissues grew well from the earliest cultures. Although there was poor survival in initial subcultures, this was attributed to the need for freshly prepared trypsin-EDTA solutions. The strong adherence of the cells to the surface of the flask, in comparison with other fish cells, resulted in longer periods of treatment with the trypsin-EDTA solution, and the result was poorer cell survival. We found this problem could be alleviated by using freshly prepared solutions of the enzyme and chelating agent.

There have been no indications of crisis episodes in the WSH-1 and WSS-2 lines up to the present passage numbers of 43 and 55, respectively. This rapid adaptation to in vitro growth may be due in part to an early development of aneuploidy as shown by the hypotetraploid nature of both sturgeon lines (modal chromosome numbers of 237 and 243 for WSH-1 and 219 for WSS-2). Although the normal 2n complement for white sturgeon is unknown, Ohno et al. (1969) found a mode of 112 chromosomes in shovel nose sturgeons *Scaphirhynchus platorhynchus*. Ohno et al. (1969) also detected microchromosomes similar to those seen in the WSS-2 and WSH-1 lines. The presence of microchromosomes distinguishes white sturgeon lines from the most commonly used cells lines (of salmonid, cyprinid, percid, and ictalurid origin) in fish health laboratories. Microchromosomes are common among avian and reptilian species and have been found in holocephalan, chondrosteian and holosteian fishes (Ohno et al. 1969). Li et al. (1985) found 99–112 chromosomes in the Atlantic sturgeon SH cell line, the only other line examined from sturgeons. If we

assume that the normal 2n mode is 112 chromosomes, the SH line has remained diploid, whereas both white sturgeon lines in our study have become aneuploid.

Cell replication in the WSS-2 line was best at temperatures of 15–25°C. (Figure 2). The most rapid cell replication, however, was observed at 25°C, a temperature near the optimum water temperature (23°C) for growth of juvenile sturgeons in culture facilities (S. Hung, University of California, Davis, personal communication). Although not tested as rigorously, the WSH-1 line seems to share the same growth responses to temperature shown by the WSS-2 line (our unpublished data).

Li et al. (1985) reported a temperature range for the SH line from Atlantic sturgeon similar to that observed with the WSS-2 line in our study. They found that SH cell replication was negligible or absent at 4 and 28°C but good at 15 and 18°C. The WSS-2 cells may have a slightly wider range of temperature tolerance, as indicated by survival (in the absence of replication) at 10 and 30°C and to a lesser extent at 35°C (Figure 2).

Isozyme electrophoresis demonstrated the identity or similarity of the two sturgeon lines to their host origin (Figure 3). The best enzymes for diagnostic purposes were PGI, AK, and GL. This technique has been useful in identifying cell line origins for numerous fish species (Lannan et al. 1984; Lidgerding et al. 1984; Li et al. 1985).

Although the WSS-2 line was not susceptible to IPNV or CCV, virus replication was demonstrated for IHNV and WSIV (Table 1). The concentrations of IHNV were appreciable but less than yields obtained from the EPC line. The WSIV grew to titers of $10^{5.2}$ TCID₅₀/mL. Our previous inability to isolate WSIV from diseased sturgeon by using cell lines of salmonid, percid, ictalurid, and centrarchid origin (Hedrick et al. 1990) indicates the importance of host-specific cell lines. In the only other study with a sturgeon cell line, Li et al. (1985) reported inclusion bodies following inoculation of the SH line with IPNV and the amphibian virus LT-1V. They did not, however, determine the production of infectious virus.

In conclusion, we have developed two new lines from the white sturgeon. One of these lines (WSS-2) may be used for virological examinations and for the propagation and study of the recently isolated WSIV. The ease with which these cells can be handled in the laboratory makes them suitable for standard laboratory procedures.

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