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FISH CELL CULTURE: CHARACTERISTICS OF A CELL LINE FROM THE SILVER PERCH, *BAIRDIELLA CHRYSURA*

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SUMMARY

A cell line designated SP-1 was established from tissue of the silver perch, *Bairdiella chrysura*. Cells were fibroblast-like and grew best at 26°C in Leibovitz medium (L-15) containing 15% fetal bovine serum and 0.150 M sodium chloride. Passage 1 to passage 9 SP-1 cells contained a chromosome number of 48; at passages 27 and 50 the modal numbers were 51 and 54, respectively. Confirmation of the origin of SP-1 cells was made by the cytotoxic antibody dye-exclusion test. This cell line supported the growth of lymphocystis virus from the silver perch but was not found to replicate various other fish and mammalian viruses.

Key words: marine teleost cell culture; *Bairdiella chrysura*.

INTRODUCTION

Fish cell culture has become an integral part of the development of an understanding of the virology, genetics and physiology of fish populations. Although a number of established fish cell lines are currently available (1), most of these lines are from freshwater fishes. Only five marine fish lines are documented, including grunt fin (GS-1) (2), Atlantic salmon (AS) (3), Omaka (4), Pacific salmon (5) and steelhead trout (5).

In this report we describe a new marine fish cell line (designated SP-1) derived from tissue of juvenile silver perch. A preliminary study indicating the usefulness of this line for lymphocystis replication has been published (6).

MATERIALS AND METHODS

Tissue culture. Twenty juvenile silver perch, 30- to 90-mm total length, were sacrificed and the swim bladders removed. The swim-bladder tissue was cut into 1-mm cubes and washed three times in Hanks' balanced salt solution containing penicillin (1000 units per ml), streptomycin (1000 mcg per ml), Fungizone (2.5 mcg per ml) (all purchased from Grand Island Biological Co.) and Gentamycin (0.1 mg per ml; Schering Corp.). Tissue cubes were planted in 30-ml plastic flasks (Falcon) (15 per flask) and overlaid with 2 ml of a growth medium consisting of L-15 (Grand Island Biological Co.) supplemented with 15% fetal bovine serum (Grand Island Biological Co.) and 1% of 1.3 M NaCl solution. Cells were incubated at 26°C and subcultured at confluency using trypsin-versene solution (ATV) (7) with a reduced versene content (1:10,000). Characterization of SP-1 cells was performed between passages 9 and 50. Presently, the line is in passage 102.

Growth studies. To characterize the growth of SP-1 cells (passage 44) 30-ml plastic flasks were seeded with 1.4×10^5 cells per ml in L-15 growth medium, and at specified intervals, cells from each of three flasks were dispersed with ATV solution and counted in a hemacytometer by trypan-blue exclusion. Additional growth parameters of SP-1 cells included basal media substitutions (passage 32; BME(H), MEM(H); Grand Island Biological Co.) and incubation temperature variation (passage 44; 18°, 32° and 37°C).

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Cell inocula and counting procedures used were the same as above.

Karyological analysis. The SP-1 line in passages 1, 9, 27 and 50 were analyzed by karyological methods (8). The primary cell analysis was required since no previous determinations of the 2N number of *Bairdiella chrysura* were available (9, 10). In calculating the mean and standard deviation, the original data were transformed using a square-root transformation. Mean values of original are shown in results (Table 1).

Cell identification. Determination of the origin of SP-1 cells was made by the cytotoxic-antibody dye-exclusion test (11) and was attempted using lactic dehydrogenase isoenzyme separation by isoelectric focusing. The isoenzyme study employed monolayers of SP-1 cells (passage 24) and various frozen (−196°C) tissues of juvenile silver perch. The separation and quantitation of lactic dehydrogenase isoenzymes were previously described (12, 13).

Virology. Naturally infected silver perch with typical lymphocystis virus (LV) lesions (14) were captured in Mississippi coastal waters. Tumor homogenates were prepared as 20% suspensions in 0.2 M phosphate-buffered saline (pH 7.0), treated with antibiotics (penicillin 100 units per ml, streptomycin 100 mcg per ml, Fungizone 0.25 mcg per ml), centrifuged to remove cellular debris and frozen at −20°C in 0.5-ml aliquots. Pools of lymphocysts tumor supernate prepared in the above manner were employed to check the susceptibility of SP-1 cells at various passage levels. Pools of LV also were prepared after one and two subcultivations in SP-1 cells and the infectivity of these pools compared to those of fresh tumor homogenates.

SP-1 susceptibility to two mammalian and four poikilothermic animal viruses was determined. These included eastern equine encephalitis (EEE), ATCC VR-65; vesicular stomatitis virus (VSV), ATCC VR-158; infectious pancreatic necrosis virus (IPN); ATCC VR-299; frog virus-3 (FV-3), ATCC VR-567; channel catfish virus (CCV), kindly supplied by Dr. J. Plumb, Auburn University, Auburn, Alabama; and spring viremia of carp virus (SVC), kindly supplied by Dr. Polly Roy, University of Alabama Medical Center, Birmingham. In each instance, SP-1 cells and known susceptible cells in 75-cm² plastic flasks were inoculated with 100 $TCID_{50}$ of virus and absorbed for 1 hr. Samples of growth fluid were taken at 0, 36 and at 72 hr or 132 hr and frozen at −60°C until analyzed. Titration of EEE and VSV was performed using Vero cells, ATCC CCL-81. CCV and IPN viruses were titrated in brown-bullhead (ATCC CCL-59) and rainbow-trout gonad (ATCC CCL-55), respectively. FV-3 and SVC were titrated in fathead-minnow cells (ATCC CCL-42).

Electron microscopy. Methods for the electron microscopic examination of normal SP-1 cells (passage 13) and cells (passage 13) infected with lymphocystis tumor homogenate were described previously (15).

Results

Tissue culture growth and cellular morphology. The SP-1 line is composed of fibroblast-like cells (Fig. 1) which are not strongly contact-inhibited. Confluent cultures of SP-1 contain between 3 and 4×10^6 cells per 25 cm² of surface area and are split weekly at a ratio of 1:8 to 1:10 using ATV solution. Cells grew best at 26° and 32°C (Fig. 2), but at the higher temperature monolayers degenerated on the 6th day. At 18°C SP-1 cell

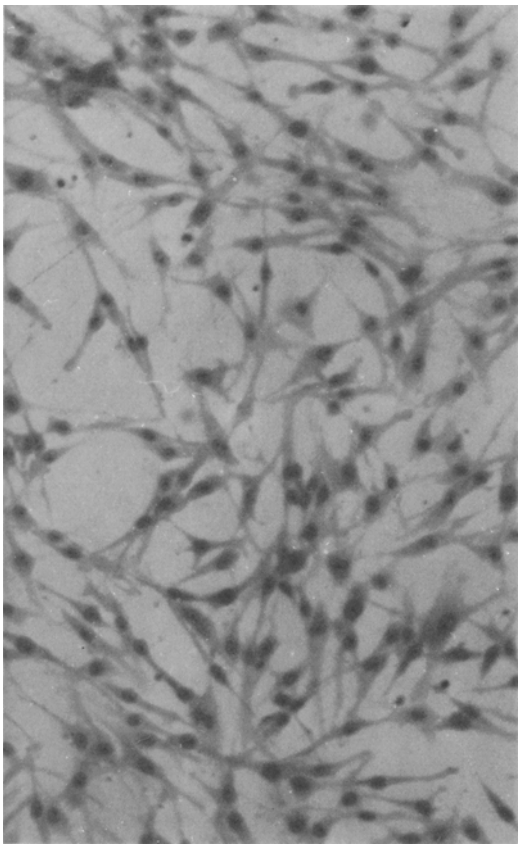

FIG. 1. Normal SP-1 monolayer. May-Grünwald-Giemsa stain. ×320.

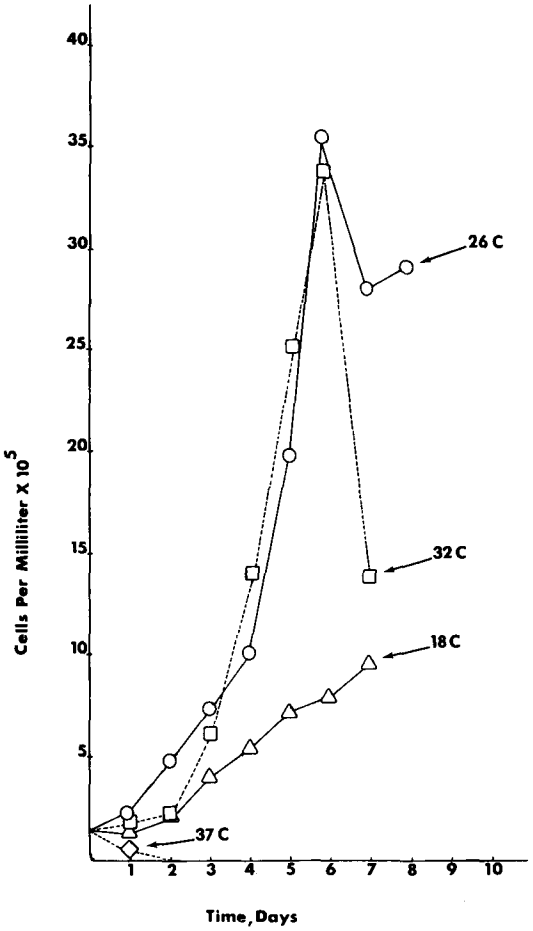

FIG. 2. Growth of SP-1 cells at temperatures of 18°, 26°, 32° and 37°C.

growth was minimal and exhibited a 6-fold increase in cell numbers in 6 days as compared to 26°C which allowed a 25-fold increase in the same length of time. Cells incubated at 37°C died rapidly (Fig. 2), and by day 2 live cells could not be detected in a hemacytometer. SP-1 cells (1.4×10^5 cells per ml) seeded into growth medium prepared by using MEM(E) or BME(H) to replace L-15 did not exhibit growth. In BME(H) cell numbers dropped to 7×10^4 cells per ml after 24 hr of incubation (26°C) and remained at less than 5×10^4 cells per ml for 6 days. Cell counts dropped, using MEM(E), to less than 5×10^4 cells per ml in 24 hr (26°C) and did not increase in number during a 5-day period.

For long-term storage, SP-1 cells were held in liquid nitrogen in L-15 growth medium containing 5% dimethylsulfoxide.

TABLE 1

KARYOLOGY AND 2N CALCULATIONS ON CELLS OF *BAIRDIELLA CHRYSURA*

		No. of Countable Spreads	x±s[a] Mean	Mode
A.[b]	Trial 1	135	47.4±0.4	48
	Trial 2	168	46.7±0.2	48
B.[c]	Passage 9	53	45.9±0.3	48
	Passage 27	53	50.7±0.4	54
	Passage 50	86	51.6±0.3	51

[a] Mean (untransformed data); standard deviation ($\sqrt{}$ transformation).

[b] Analysis of chromosomes from passage 1 silver-perch tissue.

[c] Karyology of SP-1 cells at three passage levels.

Karyology. Table 1 shows the results of chromosome analysis of primary silver-perch cells and three passage levels of the SP-1 cell line. The primary cultures were analyzed in an attempt to determine the 2N number of the silver perch. In trial 1, 135 spreads were counted with a mean of 47.4±0.4 and a mode of 48. In trial 2, 168 spreads were calculated to have a mean of 46.7±0.2 and a mode of 48.

Karyological analysis of the SP-1 cell line was performed at passages 9, 27 and 50. At passage 9, 53 spreads were counted with a mean value of 45.9±0.3 and a mode of 48. At this passage, little deviation from the analysis of the primary cells could be detected as 79.2% of the spreads fell into the 45 to 48 chromosome range, and the modal number was identical to the value calculated for primary cells.

Fifty-three spreads were counted at passage 27 and the calculated mean and mode were 50.7±0.4 and 54, respectively. At this passage only 5.7% of all spreads fell into the chromosome range of 45 to 48. At passage 50, the chromosome number again was elevated over primary and passage 9 results. The mean was calculated to be 51.6±0.3 and the mode 51. Here, 13.9% of all spreads fell into the 45 to 48 range. At passage 50, 75% of all spreads fell into a range of 49 to 56 chromosomes.

The dominant type of chromosome observed in primary and subcultured silver-perch cells was the telocentric (Fig. 3). Cells at all passage levels usually contained two single satellited telocentric chromosomes (SAT-t1). At passage 27, one spread contained a chromosome which had the morphology of a SAT-t2 (two satellites) (Fig. 3). For comparative purposed, the figure also contains a spread of SP-1, P-93 chromosomes containing four satellited chromosomes. The P-93 spread (Fig. 3) was stained in an attempt to

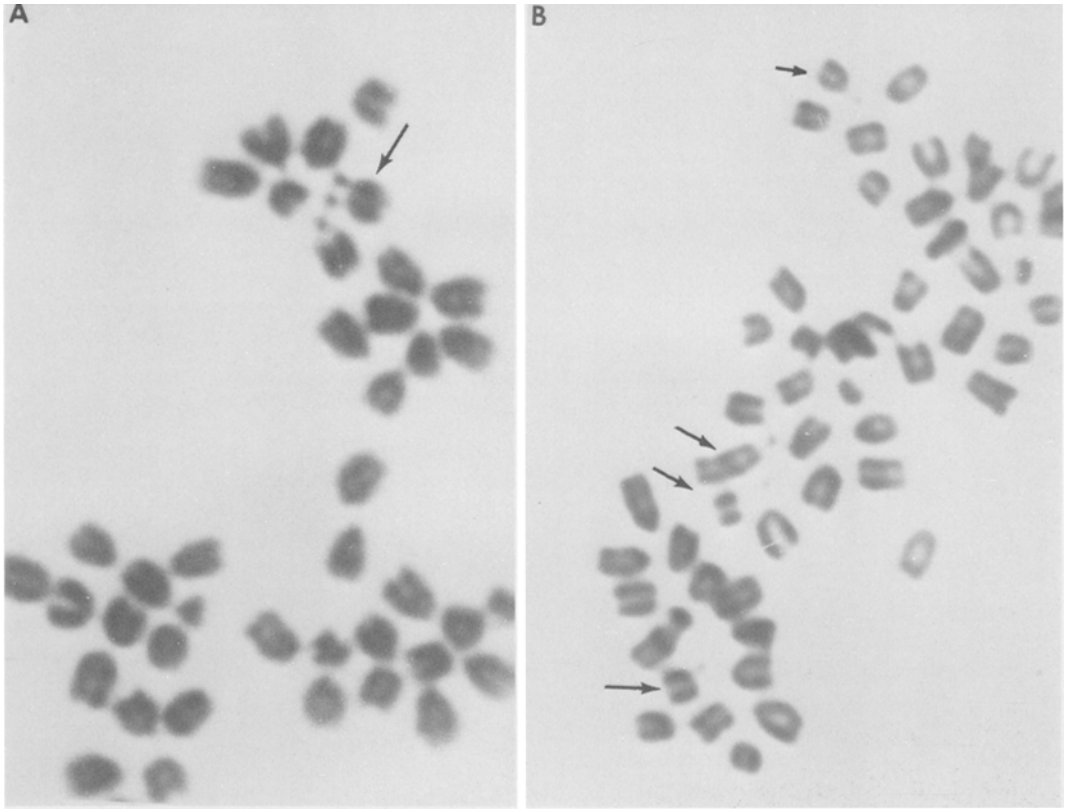

FIG. 3. Chromosome morphology of SP-1 cells in culture. *A*, SP-1, passage 27 spread containing one SAT-t1 and one SAT-t2 chromosome (*arrow*) (×4000). *B*, SP-1, passage 93 spread containing four SAT-t1 chromosomes (*arrows*) (×8000).

demonstrate the G-banding characteristics of this cell line and used a modification of the method of Deaven and Petersen (16).

Cell identification. SP-1 cells were identified immunologically using cytotoxic antibody dye exclusion, and the results of three experiments are shown in Table 2. In each case the viability of SP-1 cells in homologous antisera was less than 50% (8 and 20%, respectively) while the viability of fathead-minnow and brown-bullhead cells remained above 70%. SP-1 viability was 98% when phosphate-buffered saline was substituted for rabbit and anti-silver-perch serum and decreased to 72% when normal rabbit serum was employed.

Species confirmation also was attempted using lactic dehydrogenase isoenzymes of SP-1 cells and various organs taken from intact fish. Silver-perch tissues and the SP-1 cell line were found to contain three major LDH bands with isolelectric points in pH values of 7.05, 6.90 and 5.84, respectively. As shown in Table 3, variation occurred in the quantity of isoenzyme in each major band, and these data can be used to possibly signify the species of origin. The SP-1 cell line closely resembled silver-perch muscle tissue and not swim-bladder tissue which was explanted in the original cultures.

TABLE 2

SP-1 IDENTIFICATION BY CYTOTOXIC ANTIBODY DYE EXCLUSION

Cell line tested	Treatment	% Cell Viability		
		Trial 1	Trial 2	Trial 3
SP-1	Anti SPT[a]	8	20	8
FHM[b]	Anti-SPT	70	73	99
BB[c]	Anti-SPT	78	79	73
SP-1	PBS[d]	98	N.D.	N.D.
SP-1	NRS[f]	N.D.	72	99

[a] Rabbit antiserum to silver-perch tissue (SPT).
[b] Fathead-minnow cells (ATCC CCL-42).
[c] Brown-bullhead cells (ATCC CCL-59).
[d] Phosphate-buffered saline.
[e] Normal rabbit serum.
[f] Normal rabbit serum.
[g] Not determined.

TABLE 3

LDH ISOENZYME PROFILES OF SP-1 CELLS AND SILVER PERCH TISSUE

	Isoenzyme Bands (PI ±0.5)			
		Major bands (%)		Other (%)
Cell/Tissue	7.05	6.90	5.84	
SP-1	80	19	3	
Swim bladder	32	24	43	1
Muscle	70	20	2	9
Ovaries	21	23	56	
Spleen	24	26	49	1
Liver	10	28	62	
Heart	6	17	50	27
Gill	45	8	9	38

Virology. Lymphocystis tumor homogenate from naturally infected silver perch was used to challenge SP-1 cultures at various passage levels from 7 to 101, and the line retained its susceptibility to this virus. The rapidity with which SP-1 monolayers showed visible cytopathic effect (CPE) appeared to depend upon the concentration of infectious virus in natural tumors or the number of times that a specific preparation of LV had been passed in tissue culture. In all instances, fresh tumor material produced visible cytopathology in 4 to 13 days. Lymphocystis virus infection in SP-1 cells began with the observation of single, enlarged cells. As the infection progressed other cells in the immediate vicinity also enlarged and produced a clumping effect. Clumps of cells eventually merged to produce tumor-like masses (Fig. 4).

Fresh tumor suspensions varied in their concetration of infective virus. Homogenates containing low numbers (10^2 $TCID_{50}$ ml^{-1}) of infective units were characterized by the formation of enlarged cells in approximately 12 days. When the titer of the homogenate was 10^6 $TCID_{50}$ ml^{-1} or greater, cytopathic changes did not progress toward giant cell formation but rather were characterized by cell rounding and detachment (in 2 to 3 days) from the monolayer. These observations are consistent with the work of Sigel et al. (17).

Previous investigators observed that LV can be propagated extensively in cell culture (17, 18), but our studies were not consistent with these results.

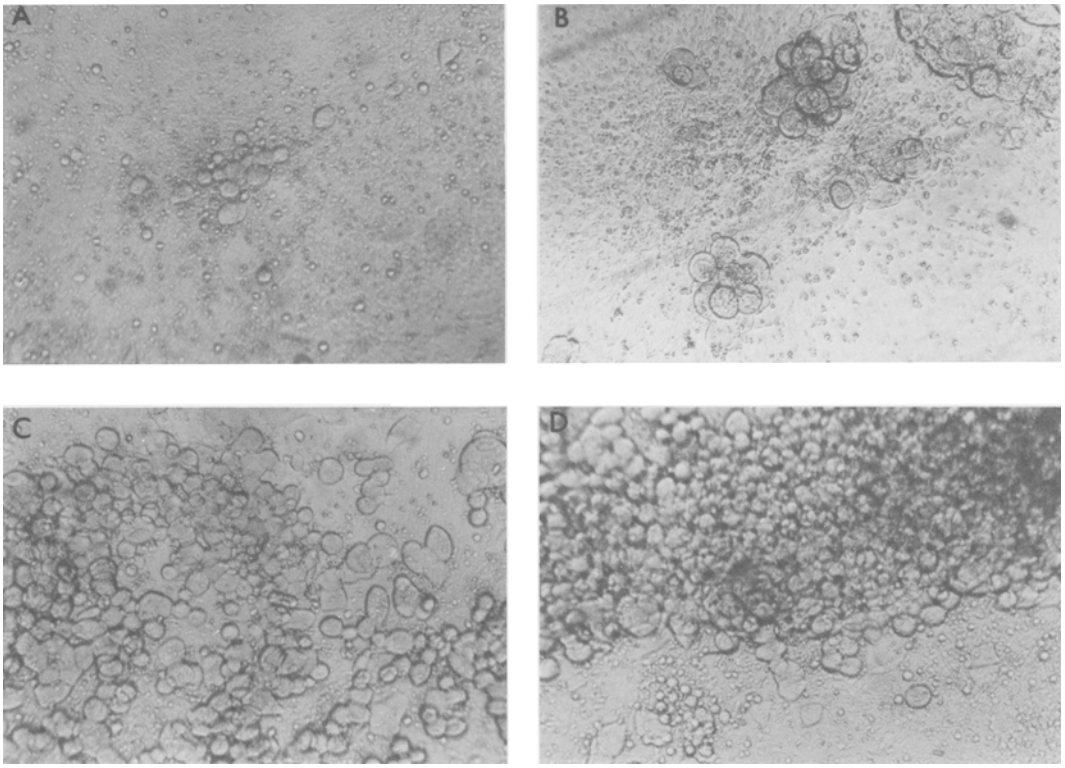

FIG. 4. SPLV in SP-1 cells. *A*, Single enlarged cells of early infection (10 days); *B*, clumps of enlarged cells (15 to 20 days); *C*, *D*, tumor-like cellular masses observed 20 to 30 days postinoculation. Unstained cells. ×75.

TABLE 4

SUSCEPTIBILITY OF SP-1 CELLS TO SEVERAL REPRESENTATIVE VIRUSES

Virus	Cell Line	Incubation Temperature	Virus Titer 0 Hr	30 Hr	72 Hr	132 Hr
IPN	SP-1	18°C	$<1\times10^2$	$<1\times10^2$	N.D.[a]	$<1\times10^2$
	RTG-2	18°C	$<1\times10^2$	1×10^3	N.D.	3×10^4
CCV	SP-1	25°C	$<1\times10^2$	$<1\times10^2$	$<1\times10^2$	N.D.
	BB	25°C	$<1\times10^2$	6.8×10^5	3.0×10^7	N.D.
SVC	SP-1	25°C	$<1\times10^2$	$<1\times10^2$	$<1\times10^2$	N.D.
	FHM	25°C	$<1\times10^2$	2.4×10^5	3.3×10^5	N.D.
FV-3	SP-1	25°C	$<1\times10^2$	$<1\times10^2$	N.D.	$<1\times10^2$
	FHM	25°C	$<1\times10^2$	1×10^3	N.D.	3.3×10^5
EEE	SP-1	33°C	$<1\times10^2$	$<1\times10^2$	$<1\times10^2$	N.D.
	VERO	33°C	$<1\times10^2$	6.8×10^4	2.9×10^5	N.D.
VSV	SP-1	33°C	$<1\times10^2$	$<1\times10^2$	$<1\times10^2$	N.D.
	VERO	33°C	$<1\times10^2$	1.2×10^6	2.4×10^6	N.D.

[a] Not determined.

As LV was passed in SP-1 cells, virus titers decreased until the fourth passage when no representative CPE was observed.

The susceptibility of SP-1 cells to two mammalian and four poikilothermic animal viruses also was examined (Table 4). In all cases, the viruses did not seem to replicate in SP-1 cells. This was not due to an inactive viral preparation nor to the temperature of incubation since each virus replicated in a chosen susceptible cell incubated at temperatures identical to those used for SP-1 cultures.

Electron microscopy. Fig. 5 demonstrates the presence of LV in a natural tumor cell of heart tissue and a cell of an infected SP-1 monolayer. In the naturally infected cell the virus arrangement was uniform and lattice-like in structure. In comparison, infected SP-1 cells contained virus particles in random arrangement. As previously reported (14), particles were icosahedral in shape, confined to the cytoplasm of the cell and measured 280 to 330 nm from vertex to vertex. Individual particles contained a nucleoid structure which was separated from the capsid by a less dense region. Capsid fragments commonly were found in SP-1 infected cells but were sparse in naturally infected cells.

DISCUSSION

Initial SP-1 cells placed in tissue culture media containing 0.137 M NaCl and 0.200 M NaCl did proliferate; however, monolayers were ragged and their integrity was lost within 3 to 4 days. Early studies compared counts of SP-1 cells grown in L-15 growth media containing 0.138 M NaCl to 0.200 M NaCl in 0.002 M increments. Cell counts performed at 30 hr postsubcultivation were tabulated and maximum growth observed between 0.146 M and 0.154 M NaCl. The 0.150 M concentration was adopted for routine propagation.

The growth of SP-1 cells at 26°C was not surprising, since this temperature is close to mean water temperatures of Mississippi estuaries during the late spring and early summer. This correlation has been observed with other poikilothermic fish cell lines (4, 5). SP-1 cells grew best in L-15 basal medium and, in contrast to other fish cell lines, did not replicate in BME(H) or MEM(E). The reason for this discrepancy is not understood.

SP-1 cells grew best in fetal bovine serum. Small concentrations of human serum (1 to 5%) did stimulate growth but were not necessary for subcultivation. Large amounts of human serum in the media (15% or greater) inhibited cell growth.

The karyological analysis of certain marine teleost cells in vitro has been reported. Regan et al. (19) analyzed primary grunt-fin tissues and determined the diploid number of *Haemulon sciurus* to be 48; however, the GF cell line contained a modal number of 46. Nicholson and Byrne (3) reported that the Atlantic-salmon (AS) cell line contained a pseudodiploid chromosome modal number of 54. The Omaka line as described by Lee and Loh (4) contained a modal number of 50 and included submetacentric and acrocentric chromosome structures.

Although relatively few marine fish have been characterized karyologically, there is reason to believe that the diploid chromosome range of 46 to 50 (4) is common and might apply to marine fish found in Gulf waters. In fact, other studies at this laboratory involving *Micropogon undulatus*, *Archosargus probatocephalus* and *Cynoscion*

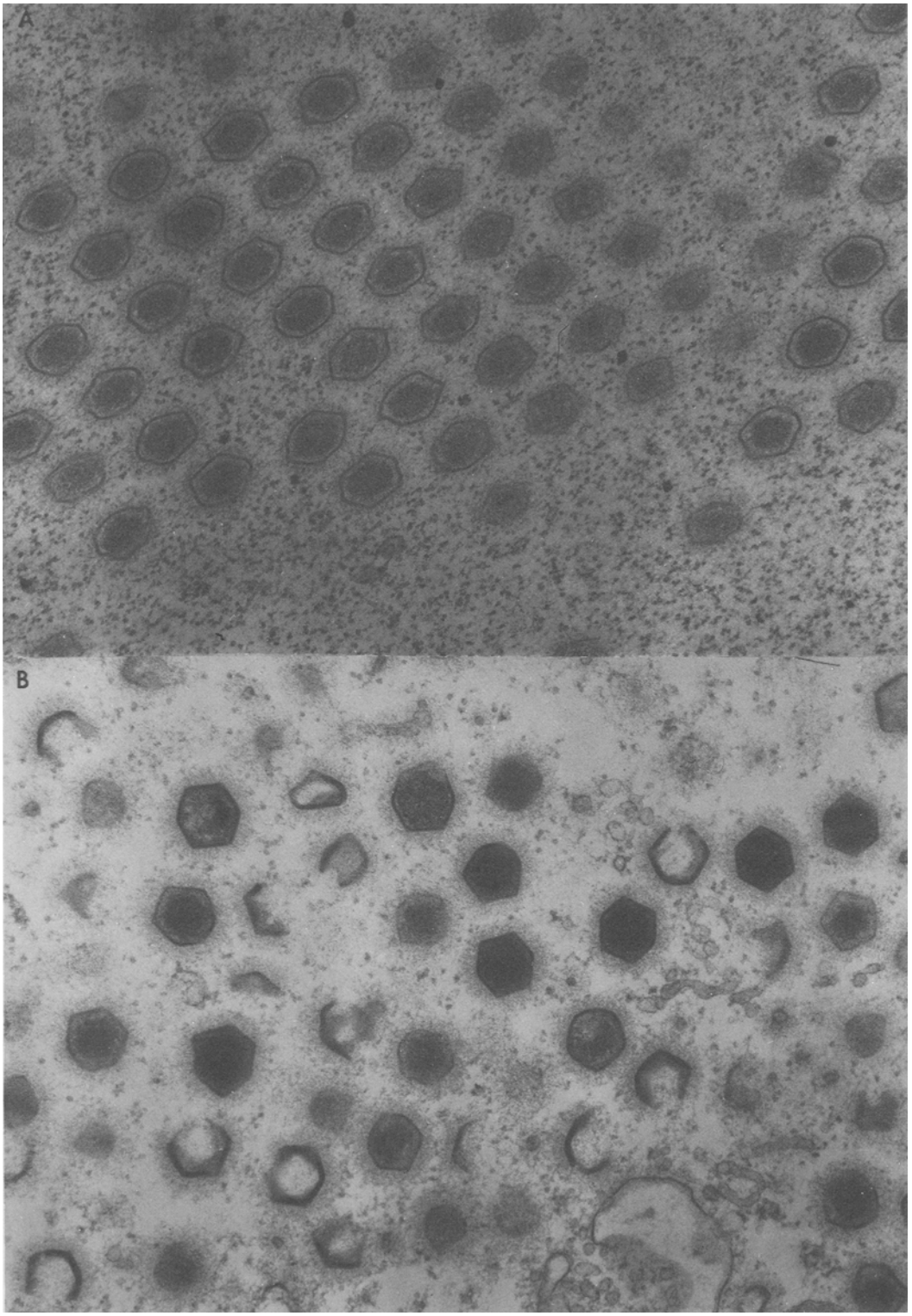

FIG. 5. Silver-perch lymphocystis virus. *A*, Natural infection of silver-perch heart tissue (×34,800); *B*, infected SP-1 cell culture (passage 13) (×34,800).

nebulosus have indicated possible diploid numbers ranging from 48 to 56.

Chromosomes of the SP-1 line followed the general pattern of previous studies in that they were small and had a tendency to adhere to one another. Rarely was chromatid material found above the centromere except in the satellited structures. Significant variation could be found in the length of the visible arms, and chromosomes often were found facing one another with no discernible connecting material. From the standpoint of cellular markers, the SAT chromosomes may be of future benefit to determine the stability of the SP-1 line. We have not ascertained if single or double satellites are the true form; however, the single satellited chromosomes were observed more frequently. The reason for the presence of this type chromosome in SP-1 cells is not understood at present.

The variation in the modal number of SP-1 cells above passage 9 was also in general agreement with previous studies. The fathead-minnow line (20) exhibited early variation in chromosome number and, at passage 50, contained a modal number of 51. Other investigators have reported that established fish cell lines showed little change from the normal karyotype (4), and, as reported by Regan et al. (19), it is possible for a Robertsonian translocation to occur in tissue culture producing a 2N number which is less than the donor species. In the case of SP-1, modal numbers calculated at passages above 9 were heteroploid. Possibly chromosome nondisjunction occurred and was selective for cells with a chromosome complement greater than 48. This theory gains additional support from the fact that the electron microscopic examination of normal SP-1 monolayers revealed that two, possibly three, different cell types existed in culture.

The use of isoelectric focusing in tissue culture characterization may be beneficial. In this study we employed only one isoenzyme marker, but specific staining methods for numerous other enzymes are available, and their analyses would allow the formation of a multi-isoenzyme profile of a cell in culture. Subsequent investigations which determine isoenzyme profiles of cultured cells from primary to established status eventually will determine the value of isoelectric focusing for cell line characterization.

The SP-1 line originally was established to replicate LV isolated from the silver perch. Lawler, Howse and Cook, in their original investigation (14), reported that LV was not only infective for external tissues of the fish, but also attacked numerous internal organs of the silver perch. Few previous reports on lymphocystis virus indicated significant internal involvement, and this LV isolate may be unique in this respect. As pointed out in our earlier report (6), SP-1 was first tested for LV susceptibility at passage 7, and subsequent studies have shown that the cell retained this susceptibility. In contrast to other lymphocystis isolates (17, 18), LV rapidly loses infectivity as it is passed in SP-1 cells, and it is not clear why this occurs. In general, this LV isolate differs from other isolates in that it is poorly preserved by lyophilization and desiccation procedures. Frozen samples (−60°C) indefinitely retain their infectivity and this is the preferred means of storage.

We have not observed the formation of the hyaline capsule of enlarged cell in vitro, although frozen LV does produce tumor cells in vivo containing this structure. Since this substance is mucopolysaccharide in nature, it may be that the L-15 growth medium does not supply the nutritional ingredients necessary for the formation of this compound. It is also possible that infected SP-1 cells in culture have lost their ability to manufacture mucopolysaccharides. Other studies at this laboratory have indicated that another marine fish cell line from tissues of the Atlantic croaker may produce hyaline capsular material in vitro when infected with LV isolated from the silver perch.

Like other marine fish cell lines, SP-1 does not appear to be susceptible to mammalian viruses; however, it differs from other established marine lines in being refractory to IPN and FV-3 (3, 4). Eastern equine encephalitis virus is known to replicate in RTG-2 cells (21) but not in GF cells (22). Its failure to replicate in SP-1 cells was not unexpected. Vesicular stomatitis virus has been cultured in poikilothermic cells (23) but not in the Omaka marine line of Lee and Loh (4). Therefore the refractory nature of SP-1 cells to VSV does not seem unusual. Future studies involving the growth of other mammalian and poikilothermic viruses in SP-1 cells may explain the lack of virus replication observed.

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