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FISH CELL LINES: TWO NEW CELL LINES DERIVED FROM EXPLANTS OF TRUNK MUSCULATURE OF *CYNOSCION ARENARIUS*

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SUMMARY

Two cell lines were established from explants of trunk musculature of healthy, males sand seatrout, *Cynoscion arenarius*. One of the lines, designated CyA-1, has been carried through 150 subcultures during 6 yr. The other, designated CyA-2, has been carried through 100 subcultures during 2 yr. Both lines grow well in L15 medium adjusted to 0.150 *M* NaCl and supplemented with 10% fetal bovine serum. Optimal growth occurs at temperatures between 24 and 30° C. The species of origin of both lines was confirmed by a cytotoxic antibody dye exclusion test. The karyotype of CyA-1 has not yet stabilized, showing a modal chromosome number of 120 at Passage 9, 89 at Passage 63, and 79 at Passage 100. The karyotype of CyA-2 is rather stable, with a modal chromosome number of 47 at Passage 1 and 49 at Passage 100. Chromosome morphology of CyA-2 is homogeneous (small, acrocentric), whereas the chromosomes of CyA-1 show considerable size variation (with small chromosomes possibly formed from fragmentation of original structures). Both lines were found to be free of bacterial or fungal contamination. Both lines supported replication of lymphocystis virus strains isolated from *Bairdiella chrysura* (the silver perch) and from *Micropogon undulatus* (the Atlantic croaker) but were refractory to 11 other viruses (4 from fish, 1 from amphibians, and 6 from mammals).

Key words: fish cell cultures; fish viruses; fish karyology.

INTRODUCTION

Lymphocystis is a viral disease affecting more than 60 species of freshwater and marine fishes. The disease is manifested by the formation of clusters of hypertrophied cells, often surrounded by a thick hyaline capsule, on the skin, fins, or internal organs. The discovery of spontaneous lymphocystis disease in several marine teleosts from the Gulf of Mexico, including *Cynoscion arenarius* and *Micropogon undulatus* (1), *Cynoscion arenarius* and *Micropogon undulatus* (1), *Cynoscion regalis* (2), and *Bairdiella chrysura* (3), suggested the need for cell lines derived from these species to facilitate studies of the virus. Existing tissue culture lines failed to support the replication of lymphocystis virus isolated from these marine species. Subsequently, several lines were developed (from marine teleosts) that were susceptible to these strains of the virus. Specifically, cell lines were developed from *B. chrysura* (4,5), *Cynoscion nebulosus* (6), and *M. undulatus* (7). Reported here are the characteristics of two new cell lines derived from one of the natural hosts for lymphocystis virus in the Gulf of Mexico, the sand seatrout (*C. arenarius*). The first line, designated CyA-1, was initiated in July 1974, and the second, designated CyA-2, was initiated in September 1978.

MATERIALS AND METHODS

Establishment of lines. The primary cell culture for CyA-1 was initiated by explant of trunk musculature from a healthy male *C. arenarius* into plastic 25 cm² flasks (Falcon Plastics, Los Angeles, CA), using Leibovitz L15 medium sup-

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plemented with 15% fetal bovine serum (FBS), 5% human serum (HuS), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml) and Garamycin (Schering Corp., Kenilworth, NJ) (0.1 mg/ml). The NaCl concentration was adjusted to 0.150 *M*. CyA-2 was initiated in an identical manner except that the serum supplement consisted of 10% FBS with no human serum. Explant cultures of both CyA-1 and CyA-2 were incubated at 28° C with weekly change of media until sufficient outgrowth of fibroblastlike cells to allow subculture was obtained (16 d for CyA-1, 30 d for CyA-2). All subcultures were made using a trypsinversene dispersant (8), with an average interval of 1 wk between subcultures using a 1:6 split ratio. Beginning at Passage 50 the serum supplement used in medium for CyA-1 was changed to 10% FBS only, and from that point on both lines were maintained on identical media.

Determination of species of origin. The species of origin of both CyA-1 and CyA-2 was confirmed using the cytotoxic antibody dye exclusion test (9). The antibody employed was prepared by injecting rabbits with a homogenate of muscle tissue from *C. arenarius*.

Sterility. For sterility testing, both lines were grown in antibiotic-free medium for 10 successive passages. The following bacteriological media were inoculated with tissue culture media from cultures afrer the 10th antibiotic-free passage: blood agar (BBL, Microbiology Systems, Becton, Dickinson & Co., Cockeysville, MD), brain heart infusion agar (Difco Laboratories, Detroit, MI), thioglycollate broth (Difco), and mycoplasma broth (BBL) enriched with 20% horse serum. Separate cultures were incubated for 10 d at 28° and 37° C. Broth cultures were incubated aerobically; agar cultures were incubated both aerobically and anaerobically.

Karyology. Chromosome analyses of CyA-1 and CyA-2 were performed at various passage levels. Preparations were made using a colcemid treatment to halt divisions in metaphase (10). One hundred chromosome spreads were counted for each cell line and passage.

Growth studies. To determine the optimum growth conditions for the lines, growth curves were developed using several variables, including temperature (18, 24, 28, 32, 34, and 37° C), FBS concentration (5, 10, 15, and 20%), HuS concentration (0 and 5%), and NaCl concentration (0.137, 0.145, 0.150, 0.177, and 0.217 *M*). In these studies, 25-cm^2 flasks (Falcon) were inoculated with a standard concentration of cells (2.5×10^5 cells/ml). Cell suspensions were prepared from two flasks daily and the number of viable cells counted with a hemocytometer using trypan blue exclusion to determine viability. The duration of these experiments was 10 d.

Plating efficiencies were determined by inoculating five flasks with CyA-1 or CyA-2 suspensions containing a total cell number previously determined using a hemocytometer. Plating efficiency was defined by the following formula: plating efficiency = number clones/number of cells in inoculum ×100.

The freeze viabilities of CyA-1 and CyA-2 were determined in growth medium with either 10% dimethyl sulfoxide or 10% glycerol as cryoprotectant. Cell suspensions from both lines (2×10^6 cells/ml) were sealed in ampules and frozen in liquid nitrogen. Ampules were removed after 48 h and the number of viable cells were counted, using trypan blue exclusion cell counts as the criterion for viability.

Virus susceptibility. Susceptibility of CyA-1 and CyA-2 cells to infection was determined for six fish, one amphibian, and six mammalian viruses. The viruses included LT-1 (11), lymphocystis (three strains, one isolated from the silver perch, *B. chrysura*, another from the Atlantic croaker, *M. undulatus*, and the third from the largemouth bass, *Micropterus salmoides*), channel catfish, spring viremia of carp, infectious pancreatic necrosis, eastern equine encephalitis, vesicular stomatitis, poliovirus Type 1, coxsackievirus (Type B3), echovirus (Type 11), and herpes simplex viruses. In virus susceptibility studies, CyA-1 and CyA-2 cultures were inoculated with a virus suspension diluted to contain 100 $TCID_{50}$ (predetermined by titration in a known susceptible cell line). In the case of lymphocystis virus, strains from marine fish lines were inoculated with a homogenized 20% w/vol suspension of lymphocystic cells from infected fish. As a control, identical amounts of the respective viruses were inoculated onto cell lines known to be susceptible. Replicate CyA-1 and CyA-2 cultures were incubated at 24, 28, and 34° C and examined daily for development of cytopathic effect (CPE). Control cultures were incubated at temperatures appropriate for each particular virus-host system. After 10 d incubation (or when maximum CPE was visible), the virus titer expressed as $TCID_{50}$/ml in medium from each culture was determined following three freeze-thaw cycles.

RESULTS

Cells CyA-1 and CyA-2 have similar morphology, both lines being typically fibroblastic. The lines form dense monolayers stable for several weeks at 28° C with no feeding. Both lines showed growth at temperatures between 18 and 34° C with optimum growth occurring at 24 to 32° C. Growth occurred over the entire NaCl range employed (0.137 to 0.217 *M*), but it was more consistent at 0.145 and 0.150 *M*, and the cells appeared more uniform in morphology at these molarities. A FBS concentration of 10% produced the best combination of growth and stability of both CyA-1 and CyA-2 cultures. As of May 1980, CyA-1 had been passed 150 times and CyA-2, 100 times. Sterility testing of CyA-1 and CyA-2 at Passages 123 and 89, respectively, showed no fungal or bacterial, including mycoplasmal, growth.

The results of the cytotoxic antibody dye exclusion test showed clearly the antigenic relatedness of *C. arenarius* muscle tissue and the CyA-1 and CyA-2 lines. When exposed to rabbit antiserum to *C. arenarius* muscle tissue, only 25% of CyA-1 and 30% of CyA-2 cells survived, whereas 95% of BB cells (ATCC CCL 59, from the brown bullhead) and 84% of CyN cells (6) (derived from muscle tissue of the spotted weakfish, *C. nebulosus*) survived. In the presence of normal rabbit serum and complement, the cell survival ranged from 82 (CyN) to 100% (CyA-1 and CyA-2).

The plating efficiencies of CyA-1 and CyA-2 in standard growth medium (10% FBS) at 28° C were 37 and 38%, respectively. The percent viability of both CyA-1 and CyA-2 cells was greater than 95% after liquid nitrogen storage in medium containing 10% DMSO, but less than 70% after storage in medium containing 10% glycerol; thus DMSO is the superior cryoprotectant for these lines.

Studies of a limited number of chromosome preparations of primary cells from *C. arenarius* indicated a chromosome number between 45 and 50. When the first complete chromosome studies of CyA-1 were made at Passage 9, however, a modal number of 120 was found. The karyotype of CyA-1 was subsequently studied at various passage levels and the modal chromosome number consistently decreased. At passage 63, for example, a modal number of 89 was found, and at Passage 100, the modal number was 79. It was the unusual karyological results obtained with

TABLE 1

CHROMOSOME DISTRIBUTION IN CyA-1 AND CyA-2 CELLS

CyA-1 (Passage 9)																															
Number of chromosomes	53–100	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125									Total
Number of cells	7	2	6	1	3	3	1	1	4	2	6	5	5	6	4	4	24	6	5	2	2	1									100
CyA-1 (Passage 63)																															
Number of chromosomes	62	63	64	66	67	68	69	70	73	74	75	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93					
Number of cells	1	1	1	1	1	5	1	2	2	2	1	2	3	4	5	2	4	4	5	11	12	15	2	10	2	1					100
CyA-2 (Passage 1)																															
Number of chromosomes	34	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	66	89	92	93–120											
Number of cells	1	1	1	1	1	3	3	6	7	4	14	12	20	9	7	1	1	1	1	6											100

CyA-1 that prompted the initiation of CyA-2. Cell CyA-2 showed a modal chromosome number of 47 at Passage 1, and the karyotype has been quite stable, with a modal number of 49 at Passages 50 and 100. Table 1 shows the chromosome frequency distribution of CyA-1 at Passages 9 and 63, and of CyA-2 at Passage 1. The chromosome morphology of CyA-2 is very homogeneous; all chromosomes are acrocentric and of approximately the same size. The chromosome morphology of CyA-1 at Passage 9 was also very homogeneous. By passage 63, however, considerable size variation was observed. It is suggested that the large number of chromosomes at Passage 9 in CyA-1 may be the result of the development (for unknown reasons) of a polyploidy and that fragmentation or outright loss of chromosomes, or both, have occurred in subsequent passages, resulting in the reduced number and increased diversity of morphology of chromosomes.

The lymphocystis virus strains isolated from *B. chrysura* and from *M. undulatus* caused CPE typical of lymphocytis virus (appearance of very large refractile, spindle-shaped cells) at all incubation temperatures. It was regularly possible to make at least five serial passes of the viruses in both CyA-1 and CyA-2 at low passage levels (below 25). At higher passage levels serial passage of the viruses was successful in only 3 of 10 attempts. Both lines were refractory to the other 10 viruses tested, including the lymphocystis virus strain isolated from the freshwater fish *M. salmoides*.

Discussion

The CyA-1 and CyA-2 lines are the first to show susceptibility both to the strains of lymphocystis virus from *B. chrysura* and to that from *M. undulatus*. The lines may therefore provide the means to study the reasons for the relatively restricted host specificity of the two viral strains and to obtain a clearer understanding of the pathogenic mechanisms of the lymphocystis virus. The somewhat unusual chromosome makeup of CyA-1 has prompted its use in several karyological studies. Both lines are easily maintained and grow well over a wide range of conditions; thus they are simple and potentially quite useful tissue culture systems.

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