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Properties of an Established Cell Line from the Atlantic Croaker (*Micropogon undulatus*) (40945)

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Abstract. A fibroblast-like cell line, CrF, has been developed from muscle tissue of the marine teleost fish *Micropogon undulatus* (the Atlantic croaker). The line was initiated by subculture of cells from explanted tissue of a single male fish. The growth medium employed contained L-15 basal medium supplemented with 15% fetal calf serum, 1 to 5% human serum, and antibiotic–antimycotic mixture. The line was characterized fully and carried through 85 subcultures. Karyological analysis showed the line to be heteroploid, with a modal chromosome number of 48 at low passage levels and 49 at high levels. All chromosomes are small and telocentric. Growth characteristics were carried out under varying conditions of incubation. Optimal growth occurred at 26 to 32° in growth medium adjusted to a NaCl molarity of 0.147 to 0.177 *M* (although a wider range was tolerated). A plating efficiency of up to 30% and freeze viability rates of 65 to 70% were observed. The line was shown to be susceptible to lymphocystis virus, but refractory to infectious pancreatic necrosis virus, spring viremia of carp virus, channel catfish virus, vesicular stomatitis virus, and eastern equine encephalitis virus. The identity of species of origin was confirmed by the cytotoxic antibody dye exclusion test, and the absence of microbial contamination was determined by inoculation of appropriate microbial growth media.

The availability of susceptible fish cell lines is essential to the isolation and study of fish viruses. The present report describes properties of a new cell line derived from fin tissue of the Atlantic croaker, *Micropogon undulatus*. This line, designated CrF, was developed as a potential tool for *in vitro* studies of lymphocystis virus isolated from several species of marine fish during natural outbreaks along the Mississippi Gulf Coast. Included among these species are the Atlantic croaker itself (1), the sand seatrout (*Cynoscion arenarius*) (1), the snook (*Centropomus sp.*) (2) and the silver perch (*Bairdiella chrysura*) (3).

Materials and methods. Establishment of cell line. A mature, male croaker captured near Ocean Springs, Mississippi, was sacrificed by decapitation. Muscle tissue from the base of the dorsal and caudal fins was removed, minced into pieces approximately 2 to 4 mm in diameter, and washed several times in Hank's balanced salt solution. The tissue pieces were placed into 30-ml plastic flasks (Falcon) and overlaid with 2 ml of L-15 medium (4), supplemented with 15% fetal calf serum (FCS), 5% human serum (HuS), penicillin (1000 units/ml), gentamycin (Schering) (0.1 mg/ml), and 1% 1.3 *M* NaCl solution (bringing the net NaCl concentration to 0.15 *M*). The flasks were placed at 26° and disturbed only when necessary for medium changes (every 3 days). After 3 weeks, when several large clones of cells had developed around the tissue pieces, the large pieces were gently dislodged and discarded. The cells in the still attached clones were dispersed with a trypsin–versene solution (ATV) (5), suspended in growth medium, and planted in new flasks. As cultures became confluent, they were subcultured at a split ratio of 1:2, using ATV as the dispersant. Subsequent subcultures were done using split ratios varying from 1:2 to 1:6, and glass as well as plastic surfaces were employed for the cultures. The HuS content of growth medium was reduced to 1% at the seventh subculture. This reduced percentage was used for routine maintenance of the line and in all experiments described unless otherwise noted.

Growth studies. A number of 30-ml plastic flasks were seeded with 2.5×10^5 cells/ml in growth medium. Two flasks were removed daily and the cells were dis-

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persed with ATV and suspended in a volume of 5 ml. The number of viable cells per milliliter in each flask was determined with a hemacytometer by exclusion of trypan blue. Growth curves were done using several variables including temperature (18°, 26°, 32°, 36°), HuS content (5%, 1%), and osmolarity (0.137, 0.147, 0.150, 0.177, 0.197, 0.217 *M* NaCl).

Plating efficiencies were determined by seeding five duplicate plastic flasks with 5 ml of suspensions containing 2000 and 200 cells/ml. After 10 days, the number of clones per flask was counted by microscopic examination and the mean plating efficiency was determined based on the number of viable cells seeded.

Preservation by freezing. CrF cells were stored in liquid nitrogen in concentrations of 2×10^6 cells/ml in growth medium plus 10% dimethyl sulfoxide or 10% glycerol. Viability tests were made after various time periods, using trypan blue exclusion cell counts as the criterion for viability.

Karyological analysis. The method used for preparation and fixation of cells for chromosome analysis has been described (6).

Viruses. The susceptibility of CrF to the following viruses was determined: eastern equine encephalitis (EEE) (ATCC VR-65); vesicular stomatitis virus (VSV) (ATCC VR-158); infectious pancreatic necrosis (IPN) (ATCC VR-299); frog virus 3 (FV-3) (ATCC VR-567); spring viremia of carp virus (SVC) (supplied by Dr. Polly Roy, University of Alabama Medical Center, Birmingham); channel catfish virus (CCV) (supplied by Dr. J. Plumb, Auburn University, Auburn, Alabama); and lymphocystis virus (LV). Two isolates of LV were used, both supplied by Dr. A. Lawler, Gulf Coast Research Laboratory, Ocean Springs, Mississippi. One LV isolate was derived from lesions from the silver perch and the other from the Atlantic croaker. The method of preparing lesions homogenates has been described (7).

In susceptibility studies, CrF and known susceptible cells in 25-cm² plastic flasks were inoculated with 1000 $TCID_{50}$ of virus (with the exception of LV, which was inoculated as a 20% lesion suspension). Adsorption was allowed to proceed for 1 hr, after which any residual inoculum was removed by several washes with growth medium. After washing, fresh growth medium was added (6 ml per flask) and flasks were placed at an appropriate incubation temperature (18° for IPN; 20° for LV, SVC, FV-3; 32° for VSV, EEE). Samples were taken immediately upon incubation, on Day 3, and on Day 7. These samples were titrated by $TCID_{50}$. Titration of EEE and VSV was performed using Vero cells ATCC CCL-81. CCV and IPN were titrated in brown bullhead (BB) (ATCC CCL-59) and rainbow trout gonad (RTG-2) (ATCC CCL-55), respectively. FV-3 and SVC were titrated in fathead minnow cells (FHM) (ATCC CCL-42). Susceptibility to LV was determined by production of cytopathic effect (CPE), in CrF cells, and in SP-1 cells (7) inoculated with material from LV-infected CrF cells.

Cell identification. Confirmation of the species origin of CrF was made by the cytotoxic antibody dye exclusion test (8).

Microbial contamination. The CrF line was regularly tested and found to be free of bacterial (including mycoplasmal) or fungal contamination. Sterility tests included inoculation of microbial growth media (PPLO broth, thioglycollate broth, Sabouraud dextrose agar) and maintenance of CrF in antibiotic-free medium for several passages.

Results. Cell morphology and growth. The CrF line consists of fibroblast-like cells (Fig. 1), showing a relatively low degree of contact inhibition. Confluent cultures reach densities of 1.2×10^5 cells/cm² in plastic flasks.

Growth characteristics of CrF cells (passage 50) in the standard growth medium for the line (with 1% HuS) at several temperatures are shown in Fig. 2. Best growth was observed at 26 and 32°. No significant growth occurred at 18°. At 36°, a significant increase in cell number occurred but peaked on Day 5, much earlier and at a much lower level than at 26 or 32°. Cultures grown at 36° typically reached confluency shortly before cessation of growth, whereas those grown at 26 or 32° showed continued growth for several days after achieving confluency. Medium pH of cultures grown

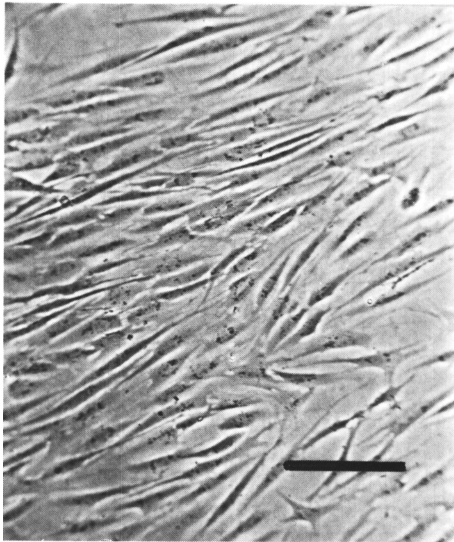

FIG. 1. Normal CrF monolayer. Phase contrast. Bar, 100 μm.

at 36° was approximately 6.8 at the time of cessation of growth, whereas in cultures grown at 26 or 32°, it was 6.5 or less. The peak cell count at 26 or 32° was not reached in the 10-day observation period. Subsequent experiments revealed that the peak occurs around Day 12. Growth curves carried out at lower passage levels (between 21 and 32) or with increased HuS content (5%)

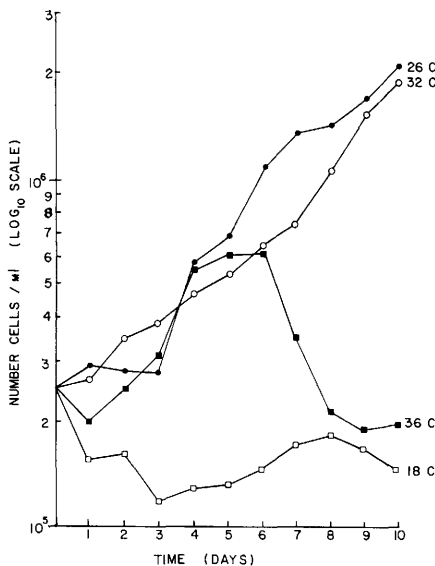

FIG. 2. Growth of CrF cells at various temperatures.

in the medium showed no significant differences from those curves shown in Fig. 2.

Figure 3 presents the results of growth studies designed to determine the optimal concentration of NaCl for CrF cells. The HuS content was 1% for these studies. The greatest cell growth occurred at a NaCl concentration of 0.177 *M*, although the molarity routinely used for CrF maintenance was 0.150 *M*. Growth was, of course, also good at the latter molarity. Significant growth occurred at all osmolarities tested, including 0.137 *M*, which is used for mammalian cell culture. Note that several of the curves were biphasic, a phenomenon which has frequently been observed in other marine fish lines developed in this laboratory (9). The reason for the apparent biphasy has not been determined. No significant differences were observed in medium pH or in the appearance of individual cells or the cell monolayer at the NaCl concentrations tested.

Determination of plating efficiencies of CrF cells was performed at several passage levels at the various temperatures used for growth studies. At 26 and 32°, the cells had plating efficiencies in the standard growth medium ranging between 22 and 30%. Plating efficiency was unmeasurable at 36 and 18°.

Viability following liquid nitrogen storage in medium containing 10% DMSO or 10% glycerol was performed at several passage levels. The two types of freeze media gave comparable results, with cell viability rates of 65 to 70%.

Cell identification. The results of the cytotoxic antibody test confirm that CrF cells are derived from *M. undulatus*. Table I summarizes these results. Undiluted antiserum to *M. undulatus* tissue reduced the viability of CrF cells to 10%, whereas 97% of the BB line survived. A dose response was seen, as increasing the antiserum dilution resulted in higher CrF viability rates. The antiserum showed little effect on heterologous cells. CrF survivial was 86% in the control where undiluted normal rabbit serum was used in place of antiserum.

Karyology. Table II shows the chromosome distribution in CrF cells at three passage levels, 17, 30, and 50. At pass 17, the

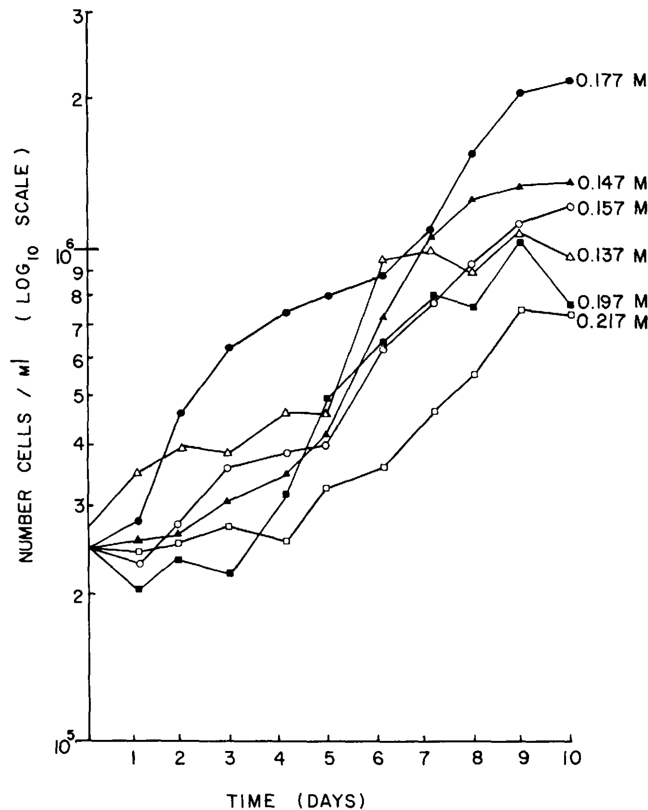

FIG. 3. Growth of CrF cells at various NaCl molarities.

modal number was 48, and at passages 30 and 50, it was 49. The modal number was found in 55% of cells at passage 17, but in only 23 and 31% of cells at passages 30 and 50, respectively. The karyogram of a typical modal cell at passage 17 consisted of 24 telocentric pairs, ranging in size from 3 to 6 μm.

Virus susceptibility. The cell lines known to be susceptible to the viruses tested dem-

TABLE I. IDENTIFICATION OF SPECIES OF ORIGIN OF CrF CELLS BY THE CYTOTOXIC ANTIBODY DYE EXCLUSION TEST

Cell line tested	Treatment	Percentage cell viability
CrF	Undiluted anti-CT[a]	10
	1/2 anti-CT	62
	1/4 anti-CT	84
	1/8 anti CT	90
	NRS[b]	86
BB[c]	Undiluted anti-CT	97
	NRS	98
STF[d]	Undiluted anti-CT	80
	NRS	88

[a] Rabbit antiserum to croaker tissue.
[b] Normal rabbit serum.
[c] Brown bullhead (ATCC CCL-5a).
[d] Speckled trout fin (a developmental cell line from *Cynoscion nebulosus*).

TABLE II. CHROMOSOME DISTRIBUTION IN CrF CELLS AT VARIOUS PASSAGE LEVELS

Passage No. 17																
Number of chromosomes	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	
Number of cells	2	3	2	2	2	3	6	6	56	13	1	2	2	1	0	N = 101[a]
Passage No. 30																
Number of chromosomes	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	
Number of cells	1	1	2	5	2	1	5	8	18	20	11	9	0	1	1	N = 85
Passage No. 50																
Number of chromosomes	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	
Number of cells	0	0	2	2	0	11	9	6	17	23	1	2	1	0	0	N = 74

[a] N, total number of cells examined.

onstrated in each case typical CPE and replication of virus to high levels. In CrF, on the other hand, the only evidence of virus susceptibility occurred in those cultures inoculated with the LV isolate from the silver perch. In these cultures grossly enlarged cells typical of LV infection began to develop by the seventh day postinoculation with maximum enlargement by day 15. Figure 4 depicts typical stained lymphocystis cells surrounded by an otherwise apparently normal CrF monolayer. The number to enlarged cells in CrF cultures infected with LV slowly increased over a 2- to 3-week period, and then began to decline. Eventually, all enlarged cells disappeared and the cultures appeared completely normal. Attempts to pass LV from CrF cells to other susceptible cultures were successful only if the inoculum was removed at the peak of the infection in the CrF cells. After the number of enlarged cells began to decline, attempts at virus passage met with increasingly limited success.

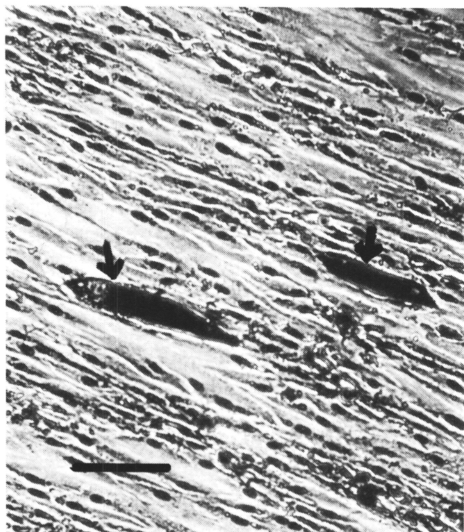

FIG. 4. Lymphocystis virus-infected CrF monolayer. Giemsa stain. Arrows indicate typical enlarged cells. Bar, 100 μm.

The failure of CrF to show virus replication or CPE when inoculated with the other viruses tested was not due to an inactive virus preparation or to incubation temperature, since each virus replicated in known susceptible cells under similar conditions. The conclusion must be that CrF is refractory to these viruses under the conditions employed.

Discussion. The present results with CrF cells are similar to those obtained with other marine fish cell lines in this laboratory (7) and other laboratories (10). Cell culture media ordinarily used for mammalian cells support the growth of these cells with only minor modifications (e.g., osmolarity). In this laboratory the use of L-15 medium is preferred since fewer pH variation problems are encountered. CrF cells seem to grow best in medium containing a small amount (1–5%) of HuS in addition to 15% FCS although they can be grown with no HuS. The optimum growth temperature for CrF cells is between 26 and 32°, correlating with the average surface temperature of water in the Gulf of Mexico in this region (23–30°).

The ability of CrF cells to grow over a wide range of osmolarity (0.137–0.217 *M* NaCl) is similar to that observed for the Omaka cell line (10) and other marine fish cell lines under development in this laboratory. Although CrF had been routinely

maintained in 0.150 *M* NaCl, best growth was obtained in 0.177 *M;* thus, this concentration is now used for routine propagation. The karyotype of CrF cells is similar to that observed in other cell lines developed from fish of the family Sciaenidae (specifically *Cynoscion nebulosus* (9), *Larimus fasciatus,* and *Bairdiella chrysura* (7)). The chromosomes are small and telocentric, and the modal numbers, 48 and 49, are within the range of diploid numbers observed for the majority of fish species (11).

The reason for the failure of CrF to support the growth of most of the viruses tested is not known but is not surprising. The virus susceptibility spectrum is identical to the SP-1 line (7) and other lines under development in this laboratory. It differs in some instances from other marine fish cell lines. For example the Omaka cell line (10) supports the growth of FV-3 and IPN, whereas CrF does not. It is surprising that the line failed to support the LV isolated from infected *Micropogon undulatus* (the homologous species) whereas it supported the *Bairdiella chrysura* isolate. No reason for this observation has been established. The *M. undulatus* isolate is capable of infecting other lines in this laboratory, for example, a line from the sand sea trout, *Cynoscion arenarius.*

The CrF line does seem well suited to the study of LV from the silver perch. The infection proceeds quickly, ample CPE develops, and new infectious virus is produced in CrF cells. The line is, moreover, easily managed in the laboratory and seems to be quite stable in culture.

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