

Some Properties of an Established Fish Cell Line from the Marine Fish, *Caranx mate* (Omaka) (38970)

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The *in vitro* cultivation of cells from different animal origins has become an invaluable part of the study of animal viruses and cell biology. Although several established fish cell lines are available for study (1), there are only a few cell lines derived from marine fishes (2-4). In the present report we have described the properties of an established cell line derived from the larvae of the marine fish, *Caranx mate*; commonly known in Hawaii as the Omaka. The Omaka is a common and excellent Hawaiian food fish and is found in protected areas such as Kaneohe Bay or Pearl Harbor off the island of Oahu. This marine teleost is classified along with the sea basses and their allies in the family Carangidae of the order Perciformes (5).

Materials and Methods. Fish larvae and cell culture preparation. Omaka larvae were collected in Kaneohe Bay off the island of Oahu, and ranged in size 1-2 cm in length (Fig. 1) which represented an age range of 2-20 days.

One hundred Omaka larvae were used to start the primary culture. The larvae were first anesthetized in cold water (4°) and the animals were sacrificed by severing the spinal cord posterior to the head and the head discarded. The decapitated larvae were thoroughly washed several times with cold Hanks' BSS and were transferred to a clean Petri dish of Hanks' BSS. After mincing up into 2 mm sq pieces the cells were dispersed with trypsin (0.25% in GKN) (0.1% glucose, 0.04% KCl, 0.8% NaCl) by mixing on a magnetic stirrer. The temperature of the solution was maintained at 24°. After 5 min, the trypsin solution was poured off as this first portion usually contains toxic substances. A fresh aliquot of trypsin was then added and the cells were dispersed on the magnetic stirrer for an additional 20 min. The cell suspension was then centrifuged (1000 rpm for 10 min) at room temperature and the pelleted cells were then resuspended in Eagle's basal

medium (EBM) (6) plus 10% fetal calf serum (EBM-10), gently triturated, and inoculated into French square bottles. The cell cultures were incubated at 22° undisturbed for 2 days, and then observed daily for cell growth.

For subcultivation primary cells grown to confluent monolayers were removed from container surfaces by either of two methods. One method required the addition of fresh EBM-10 and the cells gently scraped off with a rubber policeman. After careful trituration with a pipette to ensure uniform dispersion, the cell suspension was split into two new culture vessels. The other method employed the enzyme trypsin. The cell cultures were incubated in a 0.05% trypsin in GKN solution for 5 min at 25° to loosen the cells from the glass surface. The cell suspension was then centrifuged (1100 rpm for 2 min) and the cell pellet resuspended in EBM-10 and inoculated into new culture vessels.

During the initial efforts for the establishment of the cell culture a higher salt concentration was employed in all media and buffers as had been recommended for the optimal growth of marine fish cells (2). The concentration of sodium chloride (NaCl) added was 0.2 M in place of 0.137 M normally present. It was later determined that this increase in salt concentration was not necessary for the optimal growth of the cell and consequently the lower concentration of NaCl was used.

In early cell passages a higher concentration of penicillin (200 units/ml) and streptomycin (200 µg/ml) was used. Also, included was the fungistatic agent, fungizone (5 µg/ml). At later cell passages the concentration of both antibiotics was reduced to 100 units/ml and 100 µg/ml, respectively, and fungizone was omitted. The Omaka cell line was routinely tested and found to be negative for the presence of microbial contaminants, such as mycoplasma, bacteria, and fungi. The following sterility tests were made:

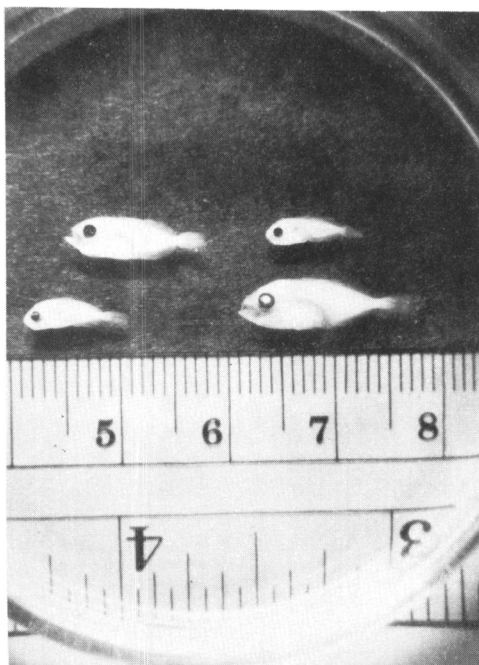


FIG. 1. Omaka fish larvae approximately 2-20 days old.

Mycoplasma: PPLO broth and PPLO agar at 37° under aerobic and anaerobic conditions.

Bacteria: Glucose broth, thioglycollate broth, marine agar, and Eagle's basal medium plus 10% fetal calf serum at 20 and 37°.

Fungi: Sabouraud's agar at 20 and 27°.

In all tests 1 ml inoculum of Omaka cell suspension from cell cultures grown in the absence of antibiotics was inoculated into each duplicate set of cultures and incubated under the appropriate conditions.

The Omaka cell has now been subcultured beyond 100 passages over a period of 30 mo. The culture still shows a mixed cell population, but with more numerous fibroblast-like cells (Fig. 2). The cells are still contact-inhibited and grow well *in vitro*. The cell characterization, as reported herein, was done between the 30-50th subculture of the cell line.

Growth studies. To determine some of the conditions for optimal growth of Omaka cells, 2-oz prescription bottles were seeded

with approximately 1.5×10^5 cells. After 1 hr attachment at room temperature and at various intervals thereafter, the number of viable cells in duplicate bottles was determined in a hemocytometer by exclusion of trypan blue. The following variables were examined: (a) temperature (16, 27, 32, and 37°); serum requirements (fetal calf serum and calf serum, at both 5 and 10% concentrations); cell media [EBM, Medium 199 (7), and NCTC 109 (8)]; and osmolarity requirements (0.137 and 0.2 M NaCl).

The method for determining the absolute plating efficiency of the cell line has been described (9).

Karyology. The method of preparation and fixation of cells for chromosomal analysis has been described (10). The karyotype of the Omaka cell was done at the 43rd cell passage.

Viruses. The susceptibility of Omaka cells to the following representative viruses was determined: the fish infectious pancreatic necrosis virus (IPN) (ATCC VR-299), the amphibian polyhedral cytoplasmic deoxyribovirus (frog virus 3, ATCC VR-567, courtesy of Dr. Allen Granoff, St. Jude Hospital, Memphis, Tenn.), vaccinia virus, reovirus type 2 (strain D-5 Jones), poliovirus type 2 and vesicular stomatitis virus (VSV). The infection procedure was as follows: Cell monolayers in Leighton tubes were challenged with virus at the following multiplicities of infection (MOI). IPN (50 TCID₅₀/cell), FV3 (0.1 PFU/cell), vaccinia (0.1 PFU/cell), reovirus type 2 [50 immunofluorescent units (IU)/cell] poliovirus type 2 (1 PFU/cell), and VSV (0.1 PFU/cell). After 1 hr adsorption at room temperature, the cells were washed with Hanks' BSS, 1 ml of EBM-1 was added per tube and incubated at 20° for IPN, 30° for FV3, and 32° for the mammalian viruses. At various intervals after adsorption, duplicate tubes for each virus system were frozen at -70° and assayed for virus at a later period.

The IPN virus was titrated in tube cultures of RTG-2 cells (rainbow trout gonads) and expressed as the 50% tissue culture infectious dose (TCID₅₀) (11). The FV3 and vaccinia viruses were assayed by the liquid-overlay

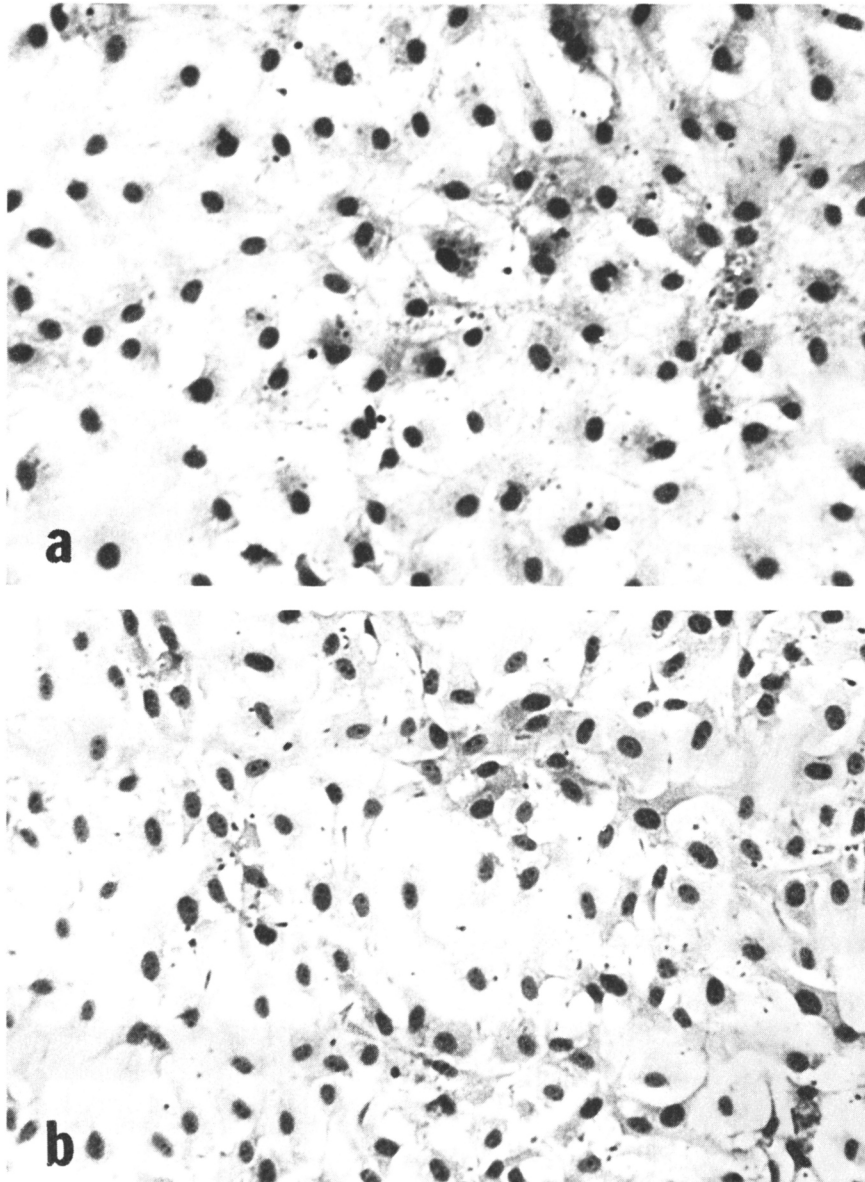


FIG. 2. Omaka cell line at different passage levels *in vitro*: (a) cells at passage 23, (b) cells at passage 63. The cells were stained with May-Grünwald Giemsa stain; $\times 530$.

plaque technique in FHM (fathead minnow) and BS-C-1 (African green monkey kidney) cell monolayers, respectively (12, 13). Reovirus type 2 was titrated by the immunofluorescent assay technique in RA cells (human amnion) (14). The poliovirus type 2 and VSV viruses were assayed by the agar-overlay plaque technique in HEP-2 (human car-

cinoma, larynx) and mouse L cells, respectively.

Induction of viral inhibitor. The synthetic double-stranded polynucleotide, polyinosinic-polycytidylic acid (poly I:C), was used to induce interferon (IF) in Omaka cells. Monolayers of Omaka cells were exposed to poly I:C (10 $\mu\text{g}/\text{ml}$) in the presence

or absence of the polysynthetization, diethylaminoethyl dextran (DEAE-dx) (100 $\mu\text{g/ml}$). Untreated cultures served as controls and all samples were incubated at 27°. After 6 and 12 hr, duplicate samples were removed and the culture fluids frozen at -70°. The fluids were later examined for IF activity by the cytopathogenic effect (CPE) reduction assay method (15) employing FV3 as the indicator virus.

Preservation by freezing. It is an advantage to store cells by freezing at extremely low temperatures. The Omaka cell line was frozen down and stored in liquid nitrogen (-196°). Briefly, confluent cell monolayers were trypsinized, pelleted and resuspended in EBM-10 to give a concentration of $2.5\text{--}3 \times 10^6$ cells/ml. Dimethyl sulphoxide (DMSO) was then added to make a 10% concentration and the cell suspension sealed in glass ampoules. After being slowly cooled at a steady rate to -20° (1°/min drop in temperature), the ampoules were rapidly transferred to the liquid nitrogen refrigerator. At certain time intervals (3, 5, and 12 mo), the cells were tested for their viability by rapidly thawing an ampoule in a 37° water bath and seeding its contents in a French square bottle containing EBM-10. After permitting the cells to attach (at 25°), the DMSO-containing medium was replaced by fresh EBM-10 and the culture observed daily for cell growth.

Results. Growth properties. The Omaka cells exhibited a maximum growth rate at 27° (Fig. 3). At 32° the cells grew slowly and eventually became granular and died. At the extreme temperatures studied, 16 and 37°, no growth was observed. However, at the lower temperature the cells remained viable and began to grow when transferred to 27°. In contrast, the higher temperature of 37° was lethal to the cells.

In determining the media and serum requirements for the growth of the Omaka cell, Medium 199 was found to provide optimal growth while EBM followed closely behind (Fig. 4). Medium NCTC 109 did not support good growth. All three media were supplemented with 10% fetal calf serum (FCS).

The source of animal sera was found to be important, as calf serum was found to be in-

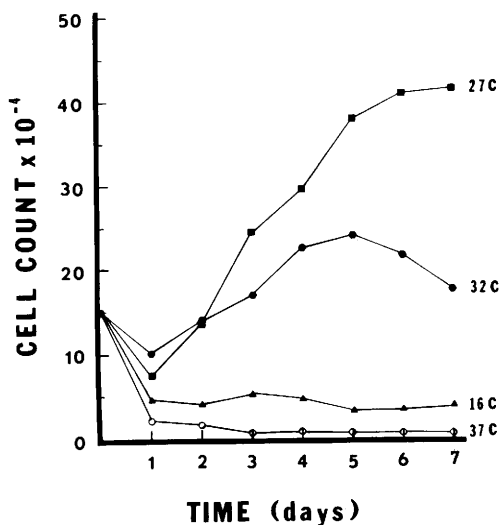


FIG. 3. Growth of Omaka cells at different incubation temperatures.

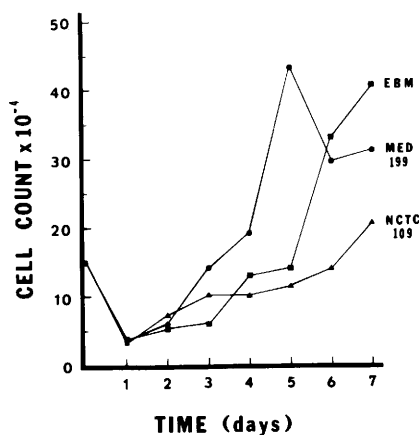


FIG. 4. Growth of Omaka cells in various tissue culture media containing 10% fetal calf serum: Eagle's basal medium (EBM) (6), Medium 199 (7), and NCTC 109 (8).

adequate as compared to FCS (Fig. 5). Furthermore, 10% FCS was found to promote better growth of the cells than 5% FCS.

A higher osmolarity requirement has been reported for the optimal growth of marine fish cells in culture (2). The effect of osmolarity in the form of a higher concentration of NaCl on the *in vitro* growth of Omaka cells was examined (Fig. 6). The results indicated that the Omaka cells did not need a high osmolarity requirement in order to grow. Growth was essentially the same at either

0.137 or 0.2 M concentration of NaCl. The former represents the concentration normally present in mammalian serum and the latter that normally present in marine fish serum.

During these growth studies, the absolute plating efficiency of the Omaka cell at different passage levels was determined. The cells had a range of 51–63 % plating efficiency.

Karyology. The karyotype of the Omaka cell line was performed at the 43rd passage *in vitro*. The chromosome distribution of 95 cells in Colcemid metaphase arrest was determined and the modal number was found to be 50 (Fig. 7). The karyogram of a modal cell consisted of 7 submetacentric pairs and 18 acrocentric pairs of chromosomes (Fig.

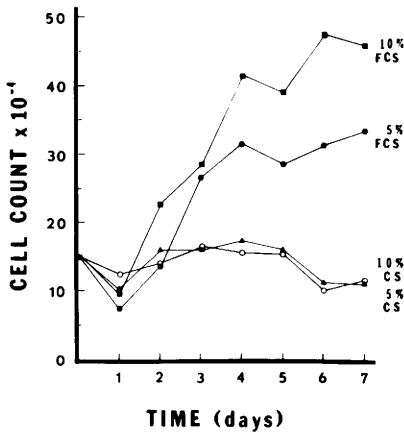


FIG. 5. Growth of Omaka cells in different concentrations of calf serum and fetal calf serum.

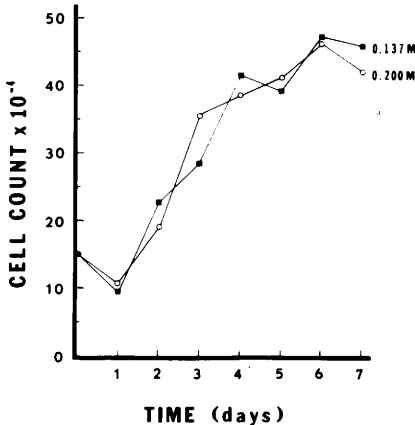


FIG. 6. Growth of Omaka cells in different concentrations of NaCl.

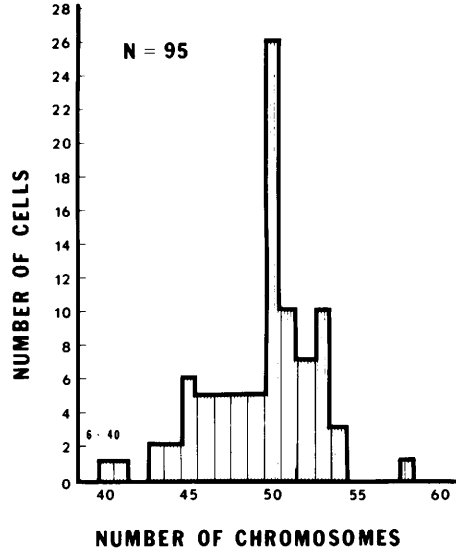


FIG. 7. Chromosome distribution of Omaka cells (passage 43). $N = 95$ = number of cells counted.

8). The chromosomes measured approximately 0.6–2 μ m in length.

Virus susceptibility. The susceptibility of Omaka cells to several representative viruses of mammalian origins and to two poikilothermic viruses was examined. The Omaka cell was found to be refractory to all of the mammalian viruses tested (Table 1). However, it supported the replication of the two poikilothermic viruses, FV3 and IPN. The latter virus did not produce any marked CPE as was observed in RTG-2 cells.

The failure of the four mammalian viruses (vaccinia, reovirus type 2, poliovirus type 2, and VSV) to replicate in Omaka cells was not due to the lower temperature selected (32°), since all mammalian viruses studied were capable of replication in either CV-1 or BS-C-1 cells at 32°.

Induction of antiviral inhibitor. A variety of fish cell lines have previously been shown to produce interferon (12, 16, 17), and it was of interest to determine whether the marine fish Omaka cells could be induced to produce this antiviral inhibitor. All attempts to induce interferon by either the synthetic polynucleotide poly I:C or poly I:C in combination with DEAE-dextran were negative and no inhibitor was detectable even at a 1:5 dilution of the culture fluids. The inhibitor

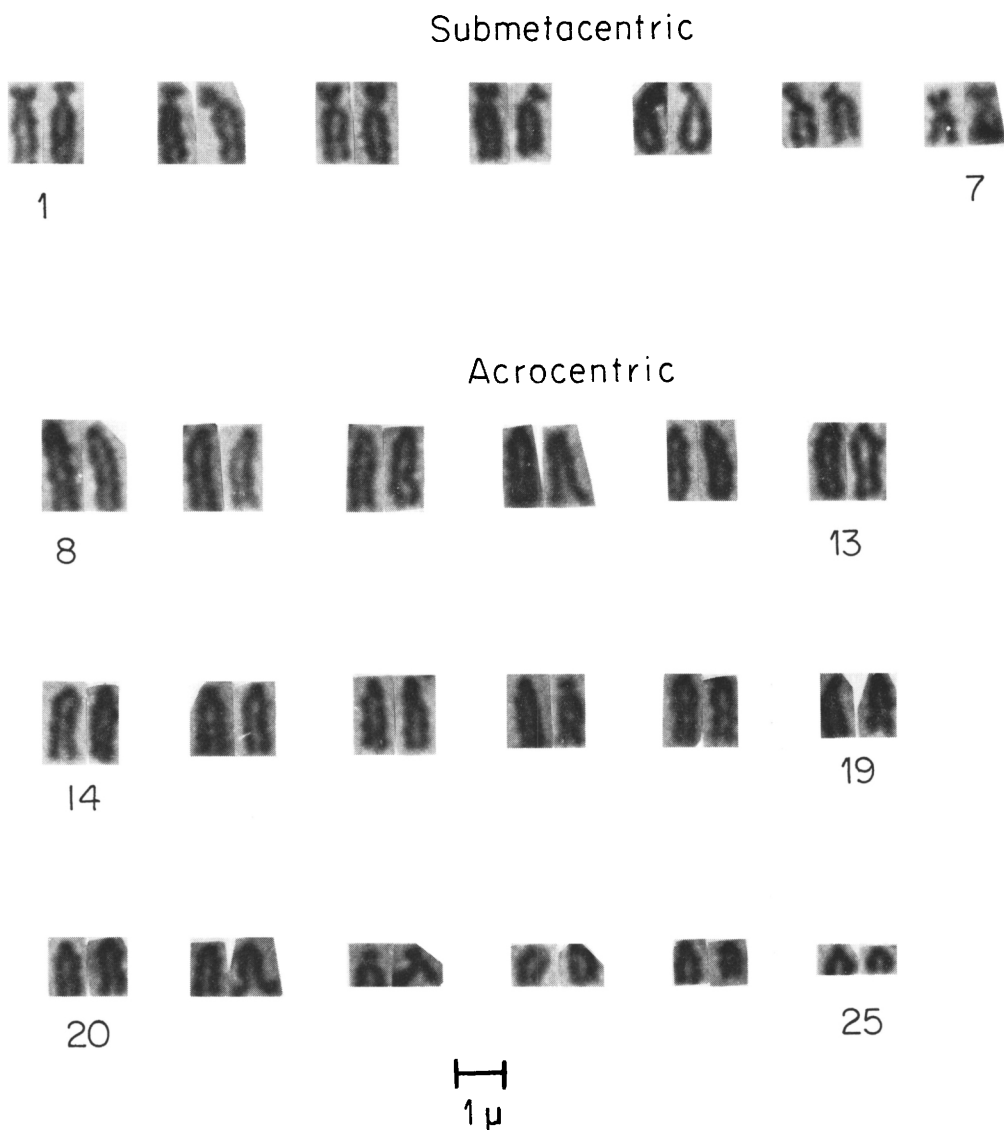


FIG. 8. A karyogram of a modal cell of Omaka passage 43. The chromosomes were tentatively paired according to size and morphology. The scale at the bottom represents 1 μ m. Giemsa stain.

was either absent or present at an extremely low level. An accompanying control cell culture of SWT (swordtail) cells treated in the same manner were found to produce an interferon-like antiviral inhibitor as was previously reported (19).

It is unlikely that Omaka cells are incapable of producing interferon for most cells of different origins have been shown to produce this inhibitor. In the present series of experiments the failure to produce interferon may

be due to either the proper inducer or conditions not having been employed. Other inducers of interferon are currently being tested.

Preservation by freezing. Methods for the storage of mammalian cells by freezing at extremely low temperatures have proven adequate for most fish cells (1). In the present experiment, Omaka cells frozen and stored by standard procedures used for mammalian cells have survived and remained vi-

TABLE I. SUSCEPTIBILITY OF OMAKA CELL LINE TO REPRESENTATIVE VIRUSES

Virus	Cell line	Virus yield ^a		
		0 hr	48 hr	96 hr
<i>Poikilothermic Viruses</i>				
FV 3	Omaka	3×10^3	4×10^4	7×10^4
	CV-1	3×10^3	1×10^5	2×10^6
IPN	Omaka	$<1 \times 10^1$	1×10^2	5×10^4
	RTG-2	1×10^2	2×10^3	2×10^3
<i>Mammalian viruses</i>				
Vaccinia	Omaka	9×10^2	3×10^2	3×10^2
	CV-1	2×10^3	1×10^6	6×10^5
Reovirus 2	Omaka	8×10^6	2×10^5	2×10^5
	CV-1	3×10^5	2×10^6	1×10^6
Poliovirus 2	Omaka	4×10^4	3×10^4	2×10^4
	CV-1	4×10^4	1×10^7	7×10^6
VSV	Omaka	9×10^1	8×10^0	$<4 \times 10^0$
	BSC-1	1×10^3	5×10^6	1×10^7

^a Virus yield is expressed in terms of PFU/ml, except for IPN which is expressed in terms of TCID₅₀/ml and reovirus 2 as IU/ml.

able during storage in liquid nitrogen (-196°) for as long as 12 mo. Upon rapid thawing and seeding, the cells grew readily into a confluent monolayer.

Discussion. The present results on the growth studies of the Omaka cells are in general agreement with previous reports on other fish cell lines (1). It is not surprising that Omaka cells grow well at 27° , as this is close to the environmental temperature of the waters of Kaneohe Bay (Oahu Island) in which the adult fish normally resides ($24-26^\circ$). This correlation between optimal temperature for fish cell culture growth and the temperature of its natural environment has been reported. Thus, cell cultures from freshwater tropical fish have been shown to grow best at temperatures around $26-27^\circ$ (18, 19). In contrast, cell cultures from fish native to colder environments, such as the salmonids, prefer temperatures around $18-24^\circ$ (3, 20).

Like other fish cell lines, the Omaka cell line grows well in cell culture media, such as Medium 199 and EBM, which are designed primarily for use with mammalian cells (2, 21, 22). Also, as with mammalian cells the substitution of fetal calf serum for calf serum yielded better growth. Calf serum in only a few cases has proven adequate for the growth of fish cells (2, 19).

The lack of effect on the growth of the Omaka cell over a wide range of osmolarity ($0.137-0.2 M$ NaCl) makes it different from the other marine fish cell line extensively studied, the GF line (grunt fin). It was reported that no primary cultures or subcultivations of the GF cell could be successfully initiated from trypsin-dispersed cells at normal levels of NaCl ($0.137 M$) (2). In the present study Omaka cells have not exhibited this strict requirement. The reasons for this difference are at present not known.

The karyotype of the Omaka cell is in general agreement with previous studies in fish karyology. The chromosomes are small as compared to those of mammals (19, 23) and the modal number of 50 falls within the diploid number found for most species of fish (46-50 chromosomes) (24). Also, it should be remembered that while *in vitro* growth has been known to cause slight alterations from the normal karyotype of fish cells (25), it has been shown that fish cell lines may become established with little or no change in chromosome karyology. The confirmation of the present diploid number of chromosomes will be sought through examination of primary Omaka cultures.

The susceptibility of Omaka cells to the poikilothermic viruses examined was not un-

expected. The FV3 virus has been reported to infect cells from a wide range of animals which includes fish, amphibians, reptiles, birds, and mammals (26). Also, the IPN virus has been reported to replicate in the majority of fish cell lines derived from different species of fishes (1).

Like the marine fish cell line, GF (27), the Omaka cell does not support the replication of the mammalian viruses tested. The refractoriness of the Omaka cell to all of the mammalian viruses examined cannot be explained solely on the basis of temperature since the corresponding control CV-1 or BS-C-1 cell cultures are able to support virus growth at 32 C. Generally, for the eurythermic cell lines, temperature appears to be a major factor in limiting replication of the mammalian and avian viruses, since the cells themselves may not survive at temperatures much above 26° (28). However, cell lines derived from salmonids can support the replication of mammalian arboviruses at low temperatures (26°) (29, 30), and VSV has been reported to replicate in SWT cells at 32° (19). The mechanism for this resistance by the Omaka cell remains to be elucidated.

Summary. A monolayer culture of fibroblast-like cells was initiated and established from a trypsin digest of Omaka fish larvae (*Caranx mate*). The cell culture has been subcultured 105 times over a period of 30 mo and appears to be contact-inhibited.

The Omaka cells grew optimally at 27° in Eagle's basal medium plus 10% fetal calf serum but failed to grow at 16 and 37°. At the lower temperature the cells remained viable and began to divide when the temperature was raised to 27°. Unlike certain marine fish cell lines the Omaka cell did not require a higher osmolarity medium for optimal growth. Karyological analysis at the 43rd passage revealed a chromosomal modal number of 50 consisting of 7 submetacentric pairs and 18 acrocentric pairs of chromosomes. Virological studies indicated that Omaka cells supported the replication of poikilothermic viruses such as, FV-3 and IPN viruses, but not the mammalian viruses examined. Attempts to induce interferon by the synthetic polynucleotide poly I:C were unsuccessful.

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