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Studies with Cells From Marine Fish in Tissue Culture.* (27059)

L. WILLIAM CLEM.† LISOLETTE MOEWUS AND M. MICHAEL SIGEL
(With Technical Assistance of John Perchalski)
Department of Microbiology, School of Medicine, University of Miami, Coral Gables, Fla.

In vitro cultivation of normal and abnormal mammalian and fowl cells has proven a valuable tool for biochemical, physiological and virological studies. The comparatively sparse work on *in vitro* cultivation of cells from fresh water fish is adequately summarized by Wolf and Dunbar(1) as is their recent work with explants of trout and goldfish tissues. More recently both Grutzner(2) and Wolf *et al.*(3) have utilized slight modifications of the technic of dispersion of cells by primary trypsinization for preparation of monolayer cultures of cells from fresh water fish. Wolf *et al.* (4) have used tissue cultures of trout cells for studying the virus causing infectious pancreatic necrosis in the young of that species. To our knowledge, there are no published reports on cultivation of tissues from marine fish. This paper describes the successful *in vitro* cultivation of cells from some marine teleost fish.

Materials and Methods. Tissues from the yellow-striped grunt (*Haemulon flavolineatum*) were used for most of this study due to their abundance in South Florida Waters, although the black angelfish (*Pomacanthus* sp.), porgy (*Calamus* sp.), pork fish (*Anisotremus virginicus*), red snapper (*Lutjanus* sp.), and sand perch (*Diplectrum formosum*) were also used at various times. These fish were all trapped in the Atlantic Ocean or in Biscayne Bay near Miami, Fla. and were kindly furnished for this study by the Miami Seaquarium. All fish were of a size to be considered sexually mature. Fish were killed just before use by severing the spinal column immediately posterior to the head. The tissues to be used were removed with sterile instruments and received three 10 minute washes at room temperature in Hanks' balanced salt solution (Hanks' BSS) containing penicillin (300 units /ml), streptomycin (150 mg/ml), and mycostatin (300 units/ml). Three to 4 explants (1-3 mm^3) per tube were cultured in stationary Bellco pyrex brand culture tubes or Leighton tubes with coverslips, usually on a coagulum of equivalent amounts of 50% chick embryo extract (prepared from 9-day-old chick embryos in Hanks' BSS) and chick plasma (Microbiological Assoc.).

Primary trypsinization was accomplished by mincing the washed tissue into small fragments which were then added to about 10 volumes of 0.25% trypsin (Difco) prepared in Mg^{++} free Hanks' BSS containing penicillin (100 units/ml) and streptomycin (50 mg/ml). In later experiments the trypsin solution contained 0.206M NaCl instead of the usual 0.137M used with mammalian systems. The trypsin was allowed to act on the tissue fragments in an Erlenmeyer flask over a magnamix at room temperature for 30 minutes after

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† U. S. Public Health Service Predoctoral Fellow.

which the initial trypsinate was discarded and fresh trypsin added. At 1 and 1½ hours the trypsin plus cells was transferred into human serum (4 volumes of trypsin plus cells and 1 volume of serum) at 5°C and then either (1) separated by centrifugation at 700 rpm for 7 minutes at 5°C and the cells resuspended in growth medium or (2) diluted with an equal volume of growth medium without centrifugation. The cells were then counted in a hemocytometer and seeded at the desired concentration in 1 ml of growth medium per culture tube. Passages were accomplished either (1) by scraping with a rubber policeman into fresh growth medium or (2) by trypsinization according to the following procedure. Four tenths ml of trypsin (0.25%) was added to each tube which was then gently agitated until the cells were off the glass (usually 3-5 minutes). Then 0.1 ml of human serum and 1.5 ml of growth medium were added to each tube. The individual cell suspensions were pooled and the cells counted. The desired cell concentration was then seeded in 1 ml of growth medium per culture tube.

Growth media used in this study were various modifications of Eagle's Basal Medium with added sera as discussed under results. Each ml of growth medium contained 100 units of penicillin, 50 mg of streptomycin, and 100 units of mycostatin. Calf serum and Eagle's concentrates (100x) were purchased from Microbiological Assoc. Human serum was purchased from Philadelphia Serum Exchange. Grunt serum was obtained by pooling the blood obtained by partial decapitation of 20 fish and separating the serum from the fibrin and cells by centrifugation in the cold. This serum was sterilized by passage through a millipore filter.

Results. Initial studies with grunt fin explants in Eagle's medium with 10% human serum showed a sequence of outgrowths which in later experiments was also seen when extra sodium chloride was added to the medium. At one day after explantation a sheet of epithelium-like cells appeared around the explant (Fig. 1). This cell type could not be subcultured and began to deteriorate after 5-8 days. A second cell type, fibroblast-like in morphology appeared at 3-5 days and after 3-5 weeks formed an extensive monolayer of cells which could be subcultured. This sequence of outgrowths occurred at 20°C and not at 30°C or 27°C. Media changes at 4-6 day intervals gave apparently the best results. No significant outgrowths occurred when calf serum was used instead of human serum. Fin explant cultures of fibroblast-like cells showed much heterogeneity between tubes but reasonably homogenous sister cultures were obtained upon successful passage (Fig. 2). Some difficulty was encountered in passing these cells and the

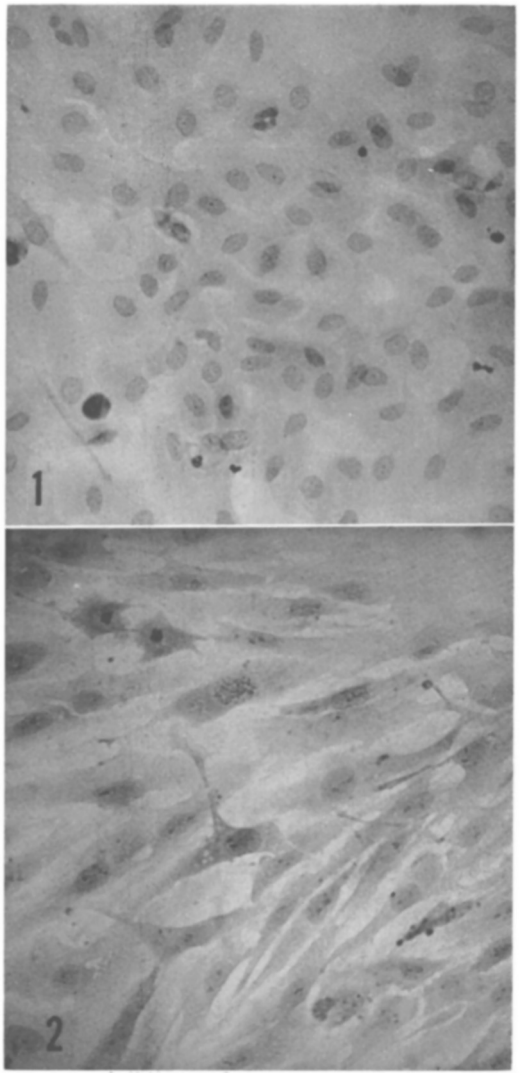

FIG. 1. Epithelium-like cell outgrowth or migration from grunt fin tissue, 3 days after explantation. H+E. 353 X.

FIG. 2. Fibroblast-like cell outgrowth from grunt fin tissue, 15 days after explantation. H+E. 353 X.

best adherence was obtained when the recipient tubes were first coated with a thin layer of plasma clot before the cells were added (about $2x10^5$ cell/ml were needed). The importance of a clot surface was evident from findings which revealed that the mere addition of chick serum and embryo extract to the medium without clot formation had no beneficial effect on cells attached directly to the glass. With all successful passages there was an increase in cell number but invariably all attempts at establishment of a cell line ended in death of the cultures, usually after 3-6 passages at 1-2 week intervals.

Explants of fin tissue from the sand perch gave similar results whereas those of the black angel fish fin failed to form monolayers of fibroblast-like cells. The first stage, migration of growth of epithelium-like cells took place. but the fibroblast-like cells which appeared toward the end of this stage did not proliferate and died after 1-2 weeks.

Grunt ovary, heart, and gill cover explants yielded monolayers of fibroblast-like cells which could be passaged. Little or no epithelium-like outgrowth appeared from these tissues. Continuous serial passages have not been undertaken. Grunt swim bladder, skin. liver, spleen, and gill explants failed to yield significant outgrowths.

Initial attempts at preparation by primary trypsinization of grunt fin cells in Eagle's medium with 10% human serum were unsuccessful. Very few cells attached to the glass or plasma clot surface and little if any growth occurred. Since the osmolarity and NaCl content of marine fish sera are higher than those of mammalian sera(5) the possibility became evident that perhaps, in contrast with organized tissue fragments, separated cells dispersed by abrupt exposure to trypsin could not adapt to the mammalian osmotic (ionic) environment as present in Eagle's medium. Typical results obtained when the NaCl content of Eagle's medium with 10% human and 10% calf was increased are shown in Table 1. These results show that the best growth was obtained when the Hanks' BSS used in preparing the growth medium contained about 0.206 molar NaCl (instead of the usual 0.137M used with mammalian cells). Subsequent studies with diluted rather than centrifuged primary cell

TABLE I. Growth of primary grunt fin cells* in Eagle's Basal Medium supplemented with 10% human serum, 10% calf serum and at various concentrations of sodium chloride.

Molar. conc. NaCl†	Condition of cultures after 13-16 days at 20°C.
.137	Few cells attached to glass surface—not proliferating.
.206	Complete monolayers of fibroblast-like cells can be successfully passaged.
.264	Incomplete monolayers of fibroblast-like cells—can be passaged later.

* These cells were separated from the trypsin by centrifugation and were seeded at 8 x 10^5 cells/ml.

† Concentration of NaCl in Hanks' Balanced Salt Solution used in preparing media.

suspensions indicated that optimal NaCl concentration was about 0.219M. Such cells gave complete monolayers of fibroblast-like cells which could be passaged at 9-10 days. These cells were morphologically indistinguishable from the fibroblast-like cells obtained from explants. In attempting further to define the requirements for culturing primary cells from a marine fish (grunt) the following observations were made. Eagle's basal medium prepared from Hanks' BSS containing 0.206M NaCl and supplemented with 10 or 20% human serum supported the growth of primary fin cells at a somewhat slower rate than did a medium of similar composition but containing a mixture of 10% human and 10% calf serum. Ten or 20% calf serum when used as the sole supplement was unsatisfactory although this medium appeaerd to be an adequate maintenance medium even without the added NaCl. The use of grunt serum with or without human or calf serum proved to be unsatisfactory for growth of primary grunt fin cells. With primary cell suspensions prepared without the use of centrifugation adequate growth could be obtained with $4\text{-}5x10^5$ cells/ml but with centrifuged suspensions growth was not obtained with seeds of less than $8x10^5$ cells/ml. Media changes at 4, 8, and 12 days yielded satisfactory results. Of the various types of culture surfaces used with primary trypsinized cells, pyrex brand culture tubes (Bellco) and plastic tissue culture bottles (Falcon) appeared most satisfactory. Soft glass prescription bottles and flint glass tubes would allow attachment and growth but the fibroblast-like cells tended to

fall back creating large holes in the cell sheet. The required temperature for growth of primary trypsinized cells was in the range of 20-22.5°C. Maximal cell yields (up to $1.5x10^6$ cells/tube) could be obtained after 3-4 weeks but most passages of primary cells were accomplished earlier (10-14 days) due to the better quality of the cells at this time. Such monolayer cultures can be serially passaged at 7-10 day intervals with 2-5 fold increases in cell number. One such line has been passaged 24 times over the past 9 months. Several passages of this line have been accomplished with seed levels as low as 25,000 cells/ml although the usual seed levels were around 10^5 cells/ml. The use of Eagle's Basal Medium supplemented with a mixture of human and calf sera with additional NaCl has proved successful for cultivation of trypsin dispersed fin cells of the porgy and the pork fish but not of the red snapper.

Discussion. The results reported here as the initial findings of a continuing study on marine life are apparently compatible in many ways with those obtained by Wolf *et al.*(1,3) for terrestrial and aquatic poikilothermic vertebrates. The basic growth sequence from explants of fin tissue of marine fish was very similar to that reported for fresh-water fish. This consisted of an initial outgrowth (or migration) of epithelium-like cells which could not be subcultured followed by appearance of a fibroblast-like cell type which was capable of being subcultured with a subsequent increase in cell number. The requirement for incubation temperatures of about 20°C and the ability to grow in media designed for mammalian cells were other points of similarity between marine and non-marine vertebrates. The important difference between the tissue culture conditions essential for cell from these 2 types of animals was the requirement for an increased sodium chloride content of the growth media when initiating primary cultures of trypsin dispersed cells from marine fish. The additional NaCl was also necessary in order to serially subculture marine fish cells; if mammalian levels of NaCl were used, the marine fish cells died after a few passages regardless of the method used to initiate them. Wolf (personal communication) has successfully subcultured fresh water trout gonad cells for over 20 passages using mammalian media. Studies are currently in progress to determine more exactly the total ionic requirements for culturing cells from marine fish, although it appears that the levels of other ions (Ca^{++}, Mg^{++}, K^+) in mammalian media are adequate to support sustained proliferation of such cells.

Since our primary objective was to develop methods for culturing cells from marine fish to be used for isolation of marine viruses, it was necessary to be able to initiate large numbers of duplicate cultures. Successful cultivation of trypsin dispersed primary cells and establishment of a serially subcultured line of cells from the grunt has facilitated production of replicate monolayers of cells not only for virus studies but also for physiological and biochemical studies. One point of interest in latter respect is the large numbers of trypsin dispersed cells required ($4x10^5$) to initiate primary cultures as compared with the much smaller number ($2.5x10^4$) which sufficed for successful subcultures. The reasons for this difference are not known but could possibly be explained on the basis of (a) the majority of the cells in the initial primary preparation are unable to divide and grow, (b) consequent selection of cells capable of *in vitro* growth under the conditions used, and (c) possible adaptation of the cells to this *in vitro* environment. On the basis of routine microscopic observation it can be stated that the initial attachment to the glass or plastic surface was poor, thereby accounting in part for some of the delay in complete monolayer formation with primary cells. The need for further studies on the nutritional aspects of marine fish cells is evidenced by the apparent species differences regarding *in vitro* growth.

Summary. Cells of tissues obtained from marine fish were shown to grow by the explant method at 20°C in a medium designed for mammalian cells. Preparation of monolayers from cells dispersed by primary trypsinization was accomplished when additional sodium chloride was added to mammalian cell media. Cells in continuous culture were found to multiply more efficiently (as regards time required for sheet formation and number of cells required for satisfactory growth) than did cells in primary culture.

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Action of Ammonium Ion on an Early Phase of Viral Infection.* (27060)

MONROE D. EATON AND ANTHONY R. SCALA
Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass.

In tumor cells influenza virus produces high titers of hemagglutinins but little infectious virus(1). This indicates an incomplete replication cycle with no spread of virus among cells. A previous report has shown that Krebs 2 ascites cells liberate ammonia from glutamine, and the antiviral effect of immediate or delayed treatment with these substances was described(2). The purpose of the investigations reported here was to obtain information about the nature of the initial process of infection during which there is a short period when viral growth may be inhibited by addition of ammonium ions to the medium. Later treatment is without effect.

Method. The medium, the method of preparing the ascites cell suspensions, and determination of cell-associated hemagglutinins have been described(2). To suspensions containing 3.2×10^6 tumor cells per ml the PR_8 strain of virus was added at a concentration of 16 to 32 hemagglutinating units (HA) per ml, giving an input multiplicity of the order of 10 E.I.D.50 per cell. Cells were allowed to adsorb virus for definite periods of time, then washed and changed to fresh medium. If a single cycle of viral replication is assumed, the HA yield reflects the proportion of cells originally infected. As judged by HA titers at 24 hours about 15% of susceptible cells were infected within 10 minutes, and virtually 100% between 2 and 4 hours. Reducing the temperature of incubation from 35°C to 4°C caused only a slight diminution in rate of viral attachment to cells. Maximum HA titers obtainable in this system were 1,000 to 2,000 units per 0.1 g of extracted cells.

Ammonium phosphate was added at a concentration of 0.01% and unless otherwise noted remained in the medium during the subsequent incubation period. Disappearance of NH_3 nitrogen from the fluid indicated an uptake by cells during the first 4 hours of about 100 μg per gram wet weight.

Results. To determine the length of the period of sensitivity to NH_4^+, cells and virus were kept at 4°C for 2 hours, then the cells were washed and suspended in medium warmed to 35°C. In the tubes receiving ammonium phosphate at 10 minutes the 24-hour HA titers were 32, in those treated at 15 minutes 128, and at 40 minutes 256 (control titer 512). This experiment shows quite clearly that at 35°C the period after adsorption during which viral growth may be significantly inhibited by NH_4^+ is only a few minutes.

When cells are kept with virus at 5°C or 15°C for a period as long as 4 hours then washed and treated immediately with NH_4^+, marked inhibition of HA formation is obtained (Table I). This indicates that low temperature prolongs the period of NH_4^+ sensitivity in contrast to its slight effect on attachment of virus. At both 25 and 35°C, however, the period of highest NH_4^+ sensitivity is about 10 to 20 minutes.

Since low temperature delays the process by which virus becomes refractory to NH_4^+, active metabolism of the host cell is probably a necessary factor in this reaction. Omitting the substrate during preincubation did not

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