GREAT LAKES FISHERY COMMISSION
Research Completion Report *

DEVELOPMENT OF LAKE TROUT CELL LINES FOR VIROLOGY RESEARCH

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Final Report for Proposal:

Development of Lake Trout Cell Lines for Virology Research

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SUMMARY

A variety of cell lines, primarily fibroblastic, were developed from lake trout of various ages. These cell lines were used for virus isolation attempts with juvenile lake trout tissue from an outbreak of epizootic epitheliotropic disease (EED) at Bayfield, WI. Discrete plaques were observed in certain cell lines inoculated with homogenates of head and kidney from affected lake trout. Cytopathic effects were also observed in lake trout cell lines inoculated with homogenates of kidney, but not spleen or abnormal lateral line obtained from lake trout with the lateral line syndrome from the Finger Lakes Cayuga and Seneca. In order to confirm viral agents as the cause of the cytopathic effects seen in lake trout cell lines, electron microscopic examination of the affected cells will be required. Such studies are in progress now.

METHODS

Cell Line Development

Tissues from lake trout of 3 ages were used in attempts to derive lake trout cell lines: 3-4 month-old fry, yearlings and 3-8 year-old adults. Fry were obtained from the Bath Hatchery, New York State Department of Environmental Conservation, Bath, NY. Yearlings were obtained from the Tunison Laboratory of Fish Nutrition, U.S. Fish and Wildlife Service, Cortland, NY. Adult lake trout were obtained during New York State Department of Environmental Conservation collections in various Finger Lakes of New York.

In initial experiments with whole fry, 5 fry were anesthetized in a solution of dechlorinated tap water containing 100-200 ppm tricaine methanesulfonate (MS 222). The fry were placed for 30 seconds in 50 ml of 1/200 Betadine povidone-iodine solution in sterile distilled water. Fish were rinsed in three 50 ml volumes of sterile phosphate buffered saline (PBS). The intestine was removed aseptically from each fish and discarded. The remaining fish bodies were placed for 5 minutes in 50 ml sterile PBS with 100 U/ml penicillin, 100 ug/ml streptomycin and 25 ug/ml Fungizone (PSF). The 5 fish bodies were then minced in a sterile petri dish using sterile scissors and forceps. The minced tissue was placed with a sterile stir bar in 10X its volume of sterile trypsin-versene (TV) solution in a sterile glass trypsinization flask. The TV solution contained 0.05%

trypsin and 0.025% versene in a PBS base. The TV-tissue suspension was gently mixed on a stir plate for 1.5-2 hr at 4 C. Following this mixing period, the cell suspension was poured through sterile cheesecloth into a sterile beaker, then placed into a sterile screw-topped polypropylene tube containing fetal calf serum (FCS) at 1/10 the volume of the TV-cell suspension. If residual large pieces of tissue remained they were again mixed for a 2-8 hr period with 10X their volume of TV at 4 C. Trypsinized cell suspensions were counted using a hemocytometer, then were centrifuged for 10 min at 200 g. Cells were seeded into 25 cm^2 flasks at 3 x 10^6 cells per flask with 5 ml of tissue culture media containing 20% FCS, PSF and 50 ug/ml gentamycin.

For yearling or adult fish, the external surface of the abdomen was swabbed with 70% ethanol. Then visceral organs (heart, gas bladder, gonad, kidney and spleen) were removed aseptically and rinsed in PBS with PSF. Surface tissues such as gill, thymus, fins, tail or neoplasms of the lateral line were flushed with 1/100 betadine solution in sterile distilled water followed by three rinses with sterile PBS with PSF. These tissues were then immersed for 5 min in 50 ml sterile PBS with PSF. Individual tissues except for lymphoid tissues were minced in sterile petri dishes. Then minced tissues were trypsinized, harvested and seeded into culture flasks as described for tissues from whole fry. Following immersion in sterile PBS with PSF, lymphoid tissues were placed in sterile polypropylene tubes containing 10 ml ice-cold tissue culture medium. Lymphoid organs were teased through 60 um mesh autoclavable screen (Tetko, Inc., Elmsford, NY) using sterile syringe plungers. Mononuclear cells in the lymphoid cell suspensions were counted using a hemocytometer and the cells were seeded into flasks as with other cell types.

In initial experiments, a variety of media and incubation temperatures were compared for culturing cells from fry and yearlings. All media used contained PSF plus 50 ug/ml gentamycin. Media tried included:

a. minimal essential medium with Hank's balanced salt solution containing 4.5 mM sodium bicarbonate (FM4) supplemented with 20% FCS (FM4-20%).

b. LM Hahn medium (which optimally supports the growth of avian lymphoblastoid cell lines) containing a mixture of Leibovitz L-15 and McCoy 5A media plus glutamine, sodium pyruvate and mercaptoethanol (Calnek et al., 1981) supplemented with either 20% FCS or 8% FCS plus 10% chicken serum.

c. Leibovitz L-15 supplemented with either 20% FCS (L-20%) or 20% FCS plus 5% lake trout serum.

d. RPMI 1640 supplemented with 20% FCS.

Incubation temperatures of 20, 18 and 15 C were tried for normal tissues from fish of the various ages. Because neoplastic cells from the lateral line grew poorly at 20C, these cells were incubated at 10, 15 and 18 C.

Cells from a yearling lake trout were cultured in FM4-20% containing 0, 100 or 1000 ng/ml phorbol myristate acetate (TPA)(Sigma) at temperatures or 15, 18 or 20 C. Cells from 4 fry were cultured in FM4-20% containing 0, 250 or 800 ng/ml of the

calcium ionophore A 23187 (Sigma). Because a significant number of cultures derived from surface tissues of fish were lost to fungal contamination, tissues from 6 fry were cultured in FM4-20% containing 2X or 3X the usual concentration of PSF in addition to 50 ug/ml of gentamycin at temperatures of either 15 or 20 C.

When primary cell lines were confluent, they were trypsinized and split 1:2 or 1:3 for subsequent passages. For the first 20 passages of any cell line, 20% conditioned medium from a confluent flask was added to the culture medium of freshly seeded cells.

We attempted to preserve several of our lake trout cell lines by freezing them in liquid nitrogen in our usual freeze medium used for standard fish cell lines. The freeze medium is comprised of 15% FCS and 7% DMSO in Leibovitz medium. Cells were sealed in sterile glass vials containing 1 ml freeze medium. Vials were held in the vapor phase over liquid nitrogen for 1 hr, then were immersed in liquid nitrogen.

Virus Isolation Attempts

Epizootic Epitheliotropic Disease Virus (EEDV)

Moribund lake trout fingerlings were collected by Sue Marcquenski, Wisconsin Department of Natural Resources, from an epidemic of EED at Bayfield Hatchery, WI. The fingerlings were placed on dry ice and shipped to Cornell University where they were stored at -90 C until assayed. Pools of tissue from groups of 5 of these fingerlings were used for virus isolation attempts. Tissues used for virus isolation were:

a. gill
b. kidney + spleen
c. liver + gut
d. head without gill.

Tissues were homogenized, centrifuged for 10 min at 500g, then supernatants were filtered through 0.45 or 0.22 um low-binding filters (Millex GV, Millipore Corporation, Bedford, MA). Aliquots of 0.2 ml of 1/100, 1/1000 or 1/10,000 dilutions of homogenates in Leibovitz-5% FCS (L-5%) were inoculated onto drained monolayers of lake trout cell lines in wells of 24-well plates. For each plate, 3 control wells were inoculated with L-5% alone and 3 positive control wells were inoculated with L-5% containing infectious pancreatic necrosis virus. Inocula were adsorbed onto monolayers for 1 hr at 15 C, then 1 ml L-5% was added per well. Inoculated plates were incubated at 10 or 15 C. Plates were examined under an inverted microscope daily during the first week, then at 2 and 3 weeks following inoculation.

Second passages were conducted with material from wells in which cytopathic effects were observed. Two techniques were used for second passages. When monolayers were desired for electron microscopy, fluid from the well was used for the second passage. In this procedure 0.5 ml aliquots from each positive well were inoculated onto fresh monolayers of lake trout cell

lines in 25 cm^2 flasks. Incubations were at 10 C and observations of monolayers were as above. If monolayers were not fixed for electron microscopy, the entire 24 well plate was frozen at -90 C, then thawed briefly to lyse cells, possibly releasing virus into the supernatants. These supernatants were then reinoculated onto fresh monolayers in 24 well plates. Plates were incubated at 10 C and observed as previously described.

Lateral Line Syndrome

Abnormal lateral line tissue, kidney and spleen were homogenized and processed as indicated above for tissues from EED cases. The same dilutions of each of these 3 tissues were inoculated onto monolayers of lake trout cell lines in 24-well plates. In addition, suspensions containing either 10^4 or 10^5 live lymphoid cells from kidney or spleen were cocultivated with monolayers of lake trout cell lines. Incubation temperatures and examination of culture plates was as indicated above for EED cases.

RESULTS

Cell Line Development

L-20% and FM4-20% were comparable and supported the best growth of lake trout cell lines among the media examined. Lake trout serum incorporated into L-20% proved toxic to lake trout cells. The phorbol ester TPA initially stimulated cell growth in primary and secondary cultures at 100 ng/ml, however, this growth advantage was no longer observed upon subsequent passages. TPA was added only to the media of primary cultures. TPA at 1000 ng/ml did not differ significantly from media without TPA. The calcium ionophore A23187 at 800 ng/ml inhibited cell growth, whereas at 250 ng/ml stimulation was not observed.

In primary and secondary cultures, most rapid growth was observed in cells from whole fry and in gonad and heart combined with gas bladder from yearling and mature fish. Unfortunately gonad and heart/gas bladder cultures did not continue to grow past the 3-5 passages. Best growth on continued passages occurred with tissues from whole fry. The next most promising results were with gill and fin tissue from yearling fish.

Lymphoblastoid cell lines were not obtained even with the TPA or calcium ionophore. Extensive effort would probably be needed to optimize the culture parameters to nurture lymphoblastoid or lymphomyeloid cell lines. Additional concentrations or combinations of TPA and calcium ionophore might be examined. Mitogen addition to the culture media might be tried. During development of avian lymphoblastoid cell lines periodic purification of viable cells from dead cells on density gradients of percoll or ficoll/hypaque are utilized. Such a strategy might aid future attempts to develop fish lymphoblastoid cell lines.

Primary cultures from neoplasms of the lateral line of 3

fish formed healthy confluent monolayers and grew well in the first few passages at 10 or 15 C. Certain cultures in the 4th passage held at 15 C remain confluent and healthy. These will be passed additional times to try to develop a continuous cell line. Cultures in the 4th passage held at 10 C all died.

Four of the most promising cultures were selected for continuing passage for development of continuous cell lines. These cultures were designated CB1G(gill), CB3(whole fry), CB5(whole fry) and CB6(whole fry) in honor of Ms. Christine Brozowski, an undergraduate student who assisted in the project. Currently CB1G cells are in the 11th, CB3 cells are in the 18th, CB5 cells are in the 7th and CB6 cells are in the 10th passage in culture. Unfortunately, various cell lines in the 4th passage did not survive freeze/thawing in liquid nitrogen.

Double and triple concentrations of PSF in the culture media did not inhibit lake trout cell growth, however, these elevated concentrations of antimicrobials also did not prevent the fungal contamination which occurred rather commonly in cultures derived from surface tissues of fish. Therefore 1X PSF was chosen for routine passing of cells. More aggressive decontamination of surface tissues with betadine or possibly another disinfectant such as benzalkonium chloride would probably prevent fungal contamination better than higher concentrations of antimycotics in culture media.

Virus Isolation Attempts

EED

Table 1 summarizes findings from virus isolation attempts using 4 separate pools of tissue each comprised of material from 5 moribund lake trout. Cytopathic effects were seen in lake trout cell lines inoculated with tissues from pools 2, 3 and 4. Homogenates of head or kidney combined with spleen most often caused cytopathic effects in cell cultures. Cell cultures were observed 1 and 2 days after inoculation and toxicity was not observed even with dilutions of 10^{-2} of the various tissues. With head and kidney/spleen from pool 2, discrete plaques were observed in cell cultures beginning at 3 days post-inoculation (dpi). Positive control wells which were inoculated with IPN showed discrete plaques initially (3-5 dpi), but by 7 dpi, the plaques spread causing severe, diffuse cytopathic effects involving the entire monolayers. In contrast, cultures inoculated with head and kidney/spleen from pool 2 retained discrete plaques throughout the month-long incubation period. Diffuse cytopathic effects (rounding up and death of cells) were observed in monolayers of CB4/5 (gill) cells inoculated with head or kidney/spleen from pool 3. Such diffuse cytopathic effects were also observed in CB3/6 monolayers inoculated with head or kidney/spleen from pool 4. In these cultures inoculated with pools 3 and 4, cytopathic effects were most severe initially around the periphery of the cell culture wells, areas in which cells were less densely packed than in the centers of the wells.

Attempts to pass the putative virus multiple times in cell

cultures failed. Therefore we have not yet been able to propagate an abundant supply of the EED virus. On a few occasions, we did succeed in demonstrating discrete plaques or diffuse cytopathic effects in monolayers in second passages of supernatants from positive culture wells. With pool 2, 0.5 ml of supernatant was removed from the positive head well, as well as from the positive kidney/spleen wells. By 4 dpi at 10 C, several discrete 1-3 mm ovoid plaques were observed in a 25 cm^2 flask containing a monolayer of CB3/3 cells inoculated with supernatant from the head well. These plaques remained discrete throughout a month-long incubation period. Unfortunately, multiple attempts to induce cytopathic effects in additional cultures with fluid from this positive flask failed. Plaques were never observed in flasks inoculated with kidney/spleen supernatant. For pool 3 the CB4/5 plate of monolayers which had been incubated at 10 C was frozen at -90 C, thawed, then 0.2 ml aliquots were adsorbed onto new CB3/4 monolayers. No toxicity was observed in these cultures 1-2 dpi. Wells (2/2) inoculated with head 10^{-2} and kidney/spleen 10^{-2} showed diffuse cytopathic effects by 7 dpi at 10 C.

Lateral Line Syndrome

Tissues from a Cayuga Lake lake trout with early lesions of the lateral line syndrome were homogenized and dilutions inoculated onto CB3/8 monolayers. Duplicate plates were prepared and incubated at either 10 or 15 C. By dpi 7 at 10 C, 2/2 wells inoculated with kidney 10^{-2} and 2/2 wells inoculated with kidney 10^{-3} showed diffuse cytopathic effects (rounding up and death of cells). Cytopathic effects were not observed in wells inoculated with spleen or lateral line incubated at 10 C nor in any wells incubated at 15 C. Second passages of supernatants from positive wells were negative on CB1G/5 (gill) or CB3/5 cells incubated at 10 C.

Tissues from a Seneca Lake lake trout with the lateral line syndrome were used to prepare single cell suspensions or homogenates for inoculation onto lake trout cell lines. Whole live kidney or spleen cells were cocultivated with monolayers of CB3/8 cells. Also homogenates of kidney, spleen or lateral line were inoculated onto monolayers of CB3/9 cells. Cytopathic effects were observed only in monolayers inoculated with homogenates of kidney cells. By dpi 4 at 10 C 2/2 wells inoculated with 10^{-2} of kidney homogenate showed diffuse cytopathic effects. Second passages of supernatants from these positive wells did not cause cytopathic effects at 10 C in CB3/5 monolayers.

DISCUSSION

Further work will be required to confirm viral agents as the cause of cytopathic effects observed in lake trout cell lines inoculated with tissues from lake trout with either EED or the lateral line syndrome. Cells from monolayers showing cytopathic effects following inoculation with these tissues are now being processed for examination under the electron microscope.

The plaques observed in CB3 monolayers inoculated with material from pool 2 were particularly striking. Thus the failure of these plaques to become confluent or to yield virus which could be readily propagated in CB3 cell lines remains perplexing. Perhaps stringent conditions are required for replication of the EED virus. In certain experiments diffuse cytopathic effects occurred in portions of CB3 monolayers which were barely confluent. This may indicate that lake trout cells must be proliferating in order to support viral replication. Since discrete plaques were observed in cell cultures of relatively low passage (2-6 passages), one might speculate that certain cell types may be required for optimal viral replication. Such cell types may have been present in initial cell cultures, but may drop out during continued passing of the various cell lines. Defective interfering particles may also play a role in limiting viral replication in cell cultures. Future Sea Grant-funded studies planned by Dr. Paul Bowser and his graduate student Li Lin Chen will attempt to optimize cell culture conditions for EED virus replication in the lake trout cell lines.

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TABLE 1: RESULTS OF INITIAL VIRUS ISOLATIONS USING TISSUES FROM MORIBUND LAKE TROUT WITH EED

Pool	Tissue	Cell Line	Incubation Temp.	Result
1	all	CB3/2	15 C	neg
1	all	CB5/2	15C	neg
2	head	CB3/2	10 C	1/1 pos(p)[a] 10^{-2}
2	kid/sp	CB3/2	10 C	2/2 pos(p) 10^{-2}
2	"	"	"	1/2 pos(p) 10^{-3}
2	all	CB5/2	10 C	neg
3	all	CB1/2[b]	10,15 C	neg
3	head	CB4/5[c]	10,15 C	1/1 pos(d)[d] 10^{-2}
3	kid/sp	"	"	2/2 pos(d) 10^{-2}
3	"	"	10 C	2/2 pos(d) 10^{-3}
3	gill	"	10,15 C	2/2 pos(p) 10^{-2}
4	head	CB3/6	10,15 C	1/1 pos(d) 10^{-2}
4	kid/sp	"	"	2/2 pos(d) 10^{-2}
4	gill	"	"	2/2 pos(p) 10^{-2}

a (p) indicates cytopathic effects characterized by discrete plaques

b CB1/2 is cell line derived from heart combined with gas bladder

c CB4/5 is cell line derived from gill

d (d) indicates cytopathic effects characterized by diffuse rounding up and death of cells in monolayer