Journal of Tissue Culture Methods **16**: 117–123, 1994.

Development of leukocyte cell lines from the channel catfish (*Ictalurus punctatus*)

Norman W. Miller, V. Gregory Chinchar & L. William Clem
Department of Microbiology, University of Mississippi Medical Center, Jackson, Mississippi, USA

Summary. Techniques are described for the generation of channel catfish long term leukocyte cell lines. These techniques include the isolation of peripheral blood leukocytes, purification of B cells by anti-immunoglobulin panning, mitogen stimulation, and in vitro maintenance and cloning of leukocyte cultures. Once stimulated in vitro, channel catfish leukocytes proliferate continuously without the need for exogenous growth factors or feeder cells. The various leukocyte cultures generated are heterogeneous and contain mixtures of monocyte/macrophage-like, T-like, or B cells. Clonal cell lines can be obtained from these mixed cultures by limiting dilution cloning in the presence of conditioned medium. A critical component of the culture medium is the use of channel catfish serum which is required for supporting and maintaining in vitro leukocyte proliferation.

Key words: B cells, T cells, Catfish, Cell lines, Leukocytes, Monocytes/macrophages

1. Introduction

Long-term cell lines from fish were first developed in the early 1960s and subsequently, a large number of such cell lines have been established from a variety of tissues and species [2, 17, 18, Fryer and Lannan, this issue]. Virtually all established fish cell lines consist of either fibroblast- or epithelial-like cells which grow as anchorage-dependent cell monolayers. These lines have proven important for the isolation and characterization of fish viruses, as well as serving as models for studies of cell function and physiology, toxicology, and carcinogenesis [10, 17]. In contrast to the success achieved with fibroblast and epithelial cultures, immunological studies in fish have been hindered by the absence of long-term leukocyte cell lines (LCLs). Recently, we found that long-term LCLs were readily generated at high frequency following in vitro stimulation of normal channel catfish peripheral blood leukocytes (PBLs) [4, 11]. These lines were anchorage-independent and could be maintained as suspension cultures without the need for exogenous factors or feeder cells. Heterogeneous LCLs containing predominantly monocytes/macrophages, T-like cells, or B cells have been generated from catfish PBLs, and clonal lines have been obtained from some cultures [4, 8, 11]. Several of these catfish LCLs have been characterized and successfully used to address various aspects of immune function [8, 11, 14]. In addition they are currently being used as in vitro models for elucidating the physiology, biochemistry, and molecular biology of fish immune responses. They are also proving useful for the study of fish immune responses following virus infection, as well as serving as valuable resources in defining intracellular events regulating productive and latent viral infections [1]. This report describes the culture system and procedures that have proven successful in the generation of catfish LCLs.

2. Materials

A. Equipment
1. CO_2 incubator, model No. 3326[1]
2. Table top centrifuge, Centra 7R, horizontal rotor No. 210[2]
3. Eppendorf microcentrifuge, model No. 5414[3]
4. Biological safety cabinet (Class 2 Type A) No. 8001-76000[4]
5. Centrifuge, superspeed, model No. RC-5B[5]
6. pH meter, Accumet 20[6]
7. Microscope, inverted, American Optical, No. 41740-020[7]
8. Spectrometer, Spectronic 601, Milton Roy, No. 57969-562[7]
9. Freezer, biological, to –85 °C, No. ULT-1985[8]

B. Chemicals
1. Tricane methane sulfonate (TMS)[9]
2. Phorbol 12-myristate-13-acetate (PMA), No. P-8139[10]
3. Calcium ionophore A23187, No. C-7522[10]
4. Gentamicin sulfate, 40 mg/ml[11]
5. Lipopolysaccharide, *S. typhimurum*, No. 3125-25[12]
6. Aim V serum-free lymphocyte medium, No. 320-2055AJ[13]
7. L-15 medium, No. 11415-023[13]

8. RPMI medium 1640 (1×), with L-glutamine, 11875-051[13]
9. Penicillin-streptomycin solution 100×, No. P-0781[10]
10. 2-Mercaptoethanol, No. M-6250[10]
11. Sodium bicarbonate, No. S-8875[10]
12. Dimethyl sulfoxide (DMSO), minimum purity 99.5% (GC), No. D-5879[10]
13. Lymphoprep, No. AN5531[14]
14. Sodium phosphate, monobasic, monohydrate No. S369[6]
15. Sodium phosphate, dibasic, anhydrous No. S374[6]
16. Sodium chloride, No. S-271[6]
17. Fetal bovine serum, Rehatuin, No. 1020-90[15]
18. Rabbit anti-mouse immunoglobulin, No. AXL-232[14]

C. Supplies

1. Pipettes, plastic disposable, serological, 1, 5, 10, and 25 ml, Nos 7075-1, 7075-5, 7075-10, and 7075-25[16]
2. Pipets, Pasteur, No. 7095B-5×[16]
3. Eppendorf digital pipetters, adjustable, 0.5–10 µl, 10–100 µl, and 100–1000 µl, Nos. 22-33-343-7, 22-33-355-1, 22-33-360-7[3]
4. Pipette tips, Nos. 53511-682 (0.5–10 µl); 53512-328 (10–100 µl), 53512-350 (100–1000 µl)[7]
5. Conical centrifuge tubes, sterile, 50 and 15 ml, Nos. 25322 and 25319-15[16]
6. Tissue culture plate, 96 well, round bottom, No. 25820-96[16]
7. Tissue culture plate, 24 well, flat bottom, No. 25820-24[16]
8. Tissue culture flask, vented, 25 cm^2, No. 25203-25[16]
9. Vacutainer blood collection tube, 10 ml, heparinized, No. 6480[17]
10. Vacutainer blood collection tube, 15 ml, no additive, No. 6432[17]
11. Vacutainer needle holder, No. 364893[17]
12. Vacutainer blood collection needles, 20 gauge × 1.5″, No. 5746[17]
13. Culture tube, sterile, snap-cap 17 × 100 mm, No. 2057[17]
14. Millex GS, Sterivex GS and Millipak 40 filter units, 0.22 µm, No. SLGS 025BS, No. SVGS 01010 and No. MPGL 04SH2[18]
15. Cryovials, 2 ml, No. 5000-0020[19]
16. Filter system, 0.22 µM cellulose acetate membrane, 200 ml, No. 25932-200[16]
17. Filter system, 0.22 µM cellulose acetate membrane, 500 ml, No. 25942-500[16]
18. Pipet Aid, Drummond, No. 53498-001[7]
19. Hemocytometer, double Neubauer, No. 15170-173[7]

3. Procedures

A. Preparation of solutions

1. Phosphate buffered saline (PBS):
   a) In 750 ml of tissue culture quality water (TCW) dissolve:
      2.56 g $NaH_2PO_4H_2O$
      11.9 g Na_2HPO_4
      87.7 g NaCl
   b) Adjust to pH 7.2.
   c) Bring volume to 1 liter with TCW.
   d) Sterilize by autoclaving 20 min at 15 lbs/in^2 and store as a 10× stock solution at room temperature.
   e) Dilute stock 1:10 with sterile TCW for 1× working solution.
2. Catfish serum:
   a) Obtain blood from the caudal sinus at the base of the anal fin from at least 10 large fish using a 20 ga. blood collection needle and 15 ml Vacutainer collection tube without additive.
   b) Allow blood to clot overnight at 4 °C.
   c) Centrifuge clotted blood at 900× g for 10 min in Centra 7R tabletop centrifuge.
   d) Pool sera and heat inactivate in a 56 °C water bath for 30 min.
   e) Centrifuge heat-inactivated sera at 13,000× g for 25 min (Sorvall RC-5B centrifuge, GSA rotor) to remove precipitated proteins.
   f) Sterilize using 0.22 µm filter and store at –20 °C in 10 ml aliquots.
3. 50 mM 2-mercaptoethanol (2-ME) solution:
   a) Add 0.35 ml 2-ME to 99.65 ml sterile TCW.
   b) Sterilize using a 0.22 µm filter and store at 4 °C in 10 ml aliquots.
   Note: Concentrated 2-ME is extremely toxic, use a chemical fume hood and do not mouth pipette.
4. Incomplete catfish medium:
   a) Add the following reagents to 80.5 ml of TCW:
      450 ml Aim V medium
      450 ml Leibovitz's L-15 medium
      5 ml 100× penicillin-streptomycin solution
      0.5 ml 40 mg/ml gentamicin sulfate solution
      1 ml 50 mM 2-ME solution (A3)
      13 ml 7.5% Na_2HCO_3 solution
   b) Adjust to pH 7.15.
   c) Sterilize using 0.22 µm filter and store at 4 °C.
5. Complete catfish medium:
   a) Add 5 ml catfish serum (A2) to 95 ml incomplete catfish medium (A4).

b) Sterilize using 0.22 μm filter and store at 4 °C.

6. Phorbol ester (PMA) solution:
   a) Dissolve PMA in DMSO at 0.5 mg/ml as stock solution.
   b) Store stock solution at –20 °C in 0.5 ml aliquots.
   c) Make working solution (1.0 μg/ml) before use by diluting the stock solution 1:500 in incomplete catfish medium (A4).
7. Calcium ionophore (A23187) solution:
   a) Dissolve the calcium ionophore A23187 in DMSO at 2.5 mg/ml as stock solution.
   b) Store stock solution at –20 °C in 0.5 ml aliquots.
   c) Make working solution (10 μg/ml) before use by diluting the stock solution 1:250 in incomplete catfish medium (A4).
8. Lipopolysaccharide (LPS) solution:
   a) Add 10 ml of sterile PBS (A1) to a vial containing 100 mg LPS.
   b) Vent vial with 20 ga needle and sterilize for 1 hour in boiling water bath.
   c) Store at –20 °C in 0.5 ml aliquots.
9. Concanavalin (ConA) solution:
   a) Dissolve ConA at 1 mg/ml in PBS (A1).
   b) Sterilize using 0.22 μm filter, and store at –20 °C in 0.5 ml aliquots.
10. Anti-mouse immunoglobulin solution:
    a) Add 1 ml of rabbit anti-mouse immunoglobulin to 39 ml PBS (A1).
    b) Adjust OD_{280} of solution to 0.3–0.4 (~0.25 mg/ml) with PBS.
    c) Sterilize by filtration through a 0.22 μm filter and store at 4 °C.
11. Murine monoclonal anti-catfish immunoglobulin antibody (mAb 9E1)
    a) Grow mouse hybridoma 9E1 at 37 °C in a humidified 5% CO_2 – 95% air atmosphere in 75 cm^2 tissue culture flask containing 30 ml RPMI-1640 supplemented with 10% fetal bovine sera.
    b) When culture becomes stationary, centrifuge for 10 min at 900× g (Centra 7R), sterilize culture supernatant using 0.22 μm filter and store in 1 ml aliquots at –20 °C.
    Note: Hybridoma 9E1 produces a murine monoclonal antibody specific for catfish immunoglobulin [5, 9]. The cell line or antibody is available from the authors upon request.
12. Cryopreservation solution:
    a) Add 1 ml DMSO to 9 ml sterile fetal bovine serum and use immediately.

B. Isolation of catfish peripheral blood leukocytes (PBLs)

1. Anesthetize a healthy catfish in dechlorinated water containing approximately 0.25 g/liter tricane methane sulfonate.
2. Obtain approximately 5–10 ml of blood from the caudal sinus at the base of the anal fin using a 20 ga collecting needle and a 10 ml heparinized Vacutainer tube.
   Note: We have found that 1 ml of blood contains $1–3 \times 10^7$ lymphocytes. In addition, up to 20 ml of blood can be taken from a 1 kg fish at 4 week intervals without harming the fish or compromising the immunological performance of the lymphocytes.
3. Dilute the blood with 2 volumes of incomplete catfish medium (A4) and carefully layer 4 ml of diluted blood over 3 ml of lymphoprep in sterile 17 × 100 mm polystyrene tubes.
   Note: Except where noted, all procedures utilizing catfish PBLs were performed at room temperature.
4. Centrifuge at 350× g for 20 min at room temperature in a Centra 7R tabletop centrifuge using horizontal rotor No. 210.
5. Remove leukocytes from the Lymphoprep interface with a sterile Pasteur pipet.
6. Wash lymphocytes by diluting isolated cells to 40 ml with incomplete medium (A4) in a sterile 50 ml conical centrifuge tube and centrifuging at 600× g for 10 min at room temperature (Centra 7R centrifuge).
7. Decant wash medium. The cell pellet is now ready for resuspension in complete catfish medium (A5) or for treatment with monoclonal antibody for B cell isolation as described below.

C. Mitogenic stimulation and culture of unfractionated PBLs

1. Suspend lymphoid cell pellet from step B7 in complete catfish medium (A5) at a cell density of $2.5–5.0 \times 10^6$ cells/ml.
2. Add 1 ml of cell suspension per well of a 24-well tissue culture plate. Alternatively, add 5 ml of cell suspension to a 25 cm^2 tissue culture flask.
3. Add 50 μl each of the PMA (A6) and A23187 (A7) working solutions per ml of cell suspension.
   Note: The final concentrations of PMA and A23187 are 0.05 μg/ml and 0.5 μg/ml, respectively. Cell suspensions can be also stimulated by adding 50 μl of LPS solution (A8) or 50 μl of ConA solution (A9) per ml of cell suspension giving final concentrations of 500 μg/ml and 50 μg/ml, respectively. In addition, the cell suspension can be incubated in the absence of mitogens.
4. Incubate cultures at 27 °C in a humidified 5% CO_2 – 95% air incubator.

5. After 12–20 hours carefully aspirate the media from the wells containing PMA/A23187 and replace with fresh complete catfish medium (A5). Alternatively, remove media containing PMA/A23187 by centrifugation at 350× g for 5 min at room temperature (Centra 7R centrifuge).
   Note: Removal of the PMA/A23187 after 24 hours is required because continued culture in their presence is cytotoxic. Mitogen removal is not required or recommended for cultures stimulated with either ConA or LPS.
6. Feed cells 3–5 days after stimulation by adding 1 ml of complete catfish medium (A5) to culture wells.
   Note: Within the first two days, mitogen-stimulated cultures show clusters of leukoblasts which rapidly increase in number during the next few days. In contrast, unstimulated cultures usually do not show blast formation until after 5–7 days in culture. However, once blasting occurs these cells proliferate quickly.
7. When cells become confluent transfer entire contents of culture well to a 25 cm^2 tissue culture flask containing 4 ml of complete catfish medium (A5). Add 1 ml of culture medium to empty well to expand the remaining cells.
8. Subculture when cell growth is heavy and medium begins to turn acid. For the first few weeks, passage cells at dilutions of 1:2 to 1:4, afterwards passages may be made at dilutions $\geq$ 1:10.

D. Isolation and stimulation of catfish B cells
1. Preparation of rabbit anti-mouse immunoglobulin coated plates:
   a) Add 0.5 ml of sterile anti-mouse immunoglobulin solution (A9) to each well of a 24 well culture plate and incubate at 4 °C for at least 18 hours.
   b) One hour before use remove antibody solution from wells, and wash 3 times with sterile PBS (A1).
      Note: Antibody solution may be re-used up to 7 times without affecting activity.
   c) Add 1 ml PBS (A1) containing 5% fetal bovine serum to the washed wells, incubate for 1 hour at 4 °C, and remove solution immediately before use.
2. Suspend catfish leukocytes pelleted from step B7 at approximately 10^7 cells/ml in mAb 9E1-containing culture supernatant (A11), and incubate on ice for 25 min.
3. Wash mAb 9E1-treated cells twice and resuspend at 2×10^6 cells/ml in incomplete catfish medium (A4).
4. Add 0.5 ml of cell suspension to the wells of the antibody-coated 24 well culture plate (D1) and incubate at 4 °C for 1 hour with occasional swirling.
5. Remove nonadherent cells with a sterile pasteur pipet and wash the wells three times with incomplete media.
6. Add 1 ml complete catfish medium to wells containing the adherent catfish B cells.
7. Add 50 µl of LPS solution (A8) to 1 ml cultures (500 µg/ml final concentration), and incubate at 27 °C in a humidified 5% CO_2 atmosphere.
8. Feed and passage proliferating cells as in steps C6–C8.

E. Cloning
1. Dilute cells from a rapidly growing culture to 3 cells/ml in 10 ml of conditioned medium containing 75% complete catfish medium (A5) and 25% cell-free culture supernatant from the cell line being cloned.
2. Add 100 µl of cell suspension to each well of a 96 well round bottom culture plate and incubate at 27 °C in a humidified 5% CO_2 – 95% air atmosphere.
   Note: Only a third of the wells should contain a cell. Using an inverted microscope we routinely scan the bottom of the cloning plate shortly after cell addition and mark the wells containing more than one cell so they will not be selected later.
3. Feed culture wells showing positive growth with 50 µl of conditioned medium (E1) when approximately 100 cells are present.
4. Passage cells from wells showing a visible pellet to wells of a 24 well culture plate containing 1 ml of conditioned medium (E1).
5. Feed and passage proliferating cells as in steps C6–C8.

F. Cryopreservation
1. Transfer cells in log phase growth (i.e. 5–7 ml of cell suspension in 25 cm^2 tissue culture flask) to a sterile 15 ml conical centrifuge tube and centrifuge at 600× g for 10 min at room temperature (Centra 7R centrifuge).
2. Suspend the cell pellet in 4 ml of cryopreservation solution (A12) and transfer 1 ml aliquots to sterile cryovials.
3. Place cyrovials in a foam-insulated box and place in –80 °C freezer overnight.
4. Remove samples and store in either –80 °C freezer or liquid nitrogen.

4. Discussion

The culture methods outlined above were successfully employed in our laboratory to generate long-term leukocyte cell lines from normal channel catfish PBLs at high frequency [4, 8, 11]. The ability of catfish PBLs to proliferate continuously in culture

after mitogenic stimulation is unique and contrasts with the situation seen with mammalian leukocytes where lymphoid cell lines require periodic re-stimulation with exogenous interleukins or feeder cells. Below we discuss some of the key features of these cell lines and the requirements for their generation.

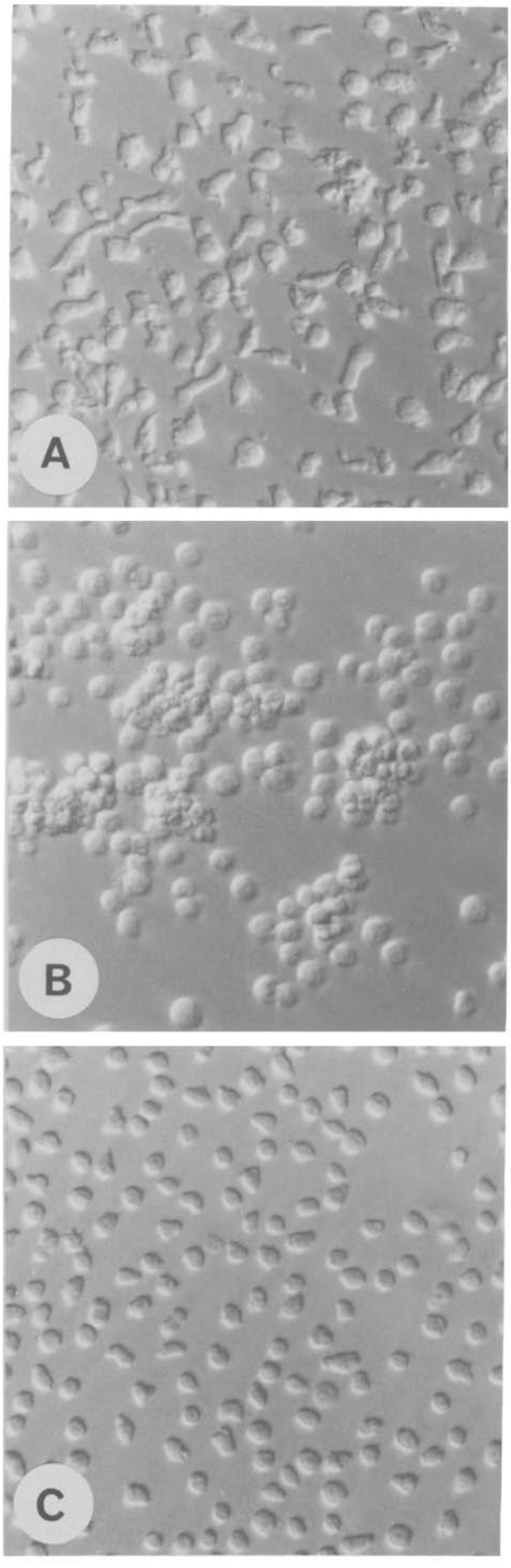
Media considerations. Optimal in vitro proliferation of channel catfish leukocytes required the correct media and sera supplements. Although fetal bovine sera (FBS) has frequently been used in the establishment of fish long-term epithelial and fibroblast cultures, its use as a media supplement for channel catfish primary leukocyte cultures has not been successful. Because of this, a culture medium consisting of a 1:1 mixture of RPMI-1640 and Click's medium adjusted to catfish tonicity and supplemented with 10% human serum and 5% catfish serum was developed and shown to support in vitro immune responses by channel catfish leukocytes [6, 7]. Recently, this medium has been replaced with one consisting of equal parts AIM-V and Liebovitz L-15 media supplemented with 5% catfish serum [8]. This change obviated the requirement for human serum, but maintained optimal in vitro proliferative responses and was subsequently used in the development of the catfish LCLs described in this report. It should be noted that catfish serum appears to be an essential component of the leukocyte culture medium. Attempts to wean established LCLs from catfish serum and into media supplemented with FBS have proven unsuccessful suggesting that one or more components present in catfish serum are required to support the growth and normal functioning of catfish leukocytes in culture.

Generation of catfish LCLs. Long-term LCLs were often established following a single exposure of catfish PBLs to mitogens. In our experience, cell cultures exhibiting a strong proliferative response shortly after mitogen treatment (induction) have a high probability of becoming established cell lines. For example, stimulation with a mixture of PMA/A23187 (a potent mitogen for catfish PBLs) resulted in the development of LCLs from greater than 95% of tested fish. In addition, stimulation with LPS and ConA were also found highly efficacious for generating LCLs [4]. However, it should also be noted that catfish LCLs have been generated from about 40% of fish in which PBLs were incubated in the absence of mitogens [4], suggesting either that a component in the medium is able to induce proliferation or that a subpopulation of catfish PBLs has the inherent ability to proliferate without stimulation. Within two days after induction, successfully stimulated cultures contained small clusters of rapidly dividing (i.e. blasting) cells which increased rapidly in size over the next few days. When the cultures became confluent, the cells were passaged at relatively low ratios (i.e. 1:2–1.4) into fresh medium. Many of these cultures continued to proliferate and have been maintained by routine subculture using high passage ratios ($\geqslant$ 1:10) for greater than two years. We suspect that as the cells became adapted to continuous culture they were less dependent upon putative growth factors present in conditioned medium and thus more able to tolerate higher dilutions during passage. Occasionally some cultures underwent a 'crisis' in which many cells began to die. In such cases, viable cells were isolated by centrifugation over Lymphoprep and re-cultured in a small volume of complete medium. This procedure has been found to induce renewed proliferation in many of the cultures and to rescue the cell line.

Long term LCLs initiated by induction with mitogens gave rise to heterogeneous cell populations consisting of T-like and monocyte/macrophage-like cells, whereas those initiated in the absence of mitogen contained predominantly monocytes/macrophages. Because B cells were rare in spontaneous and PMA/ionophore-induced cultures, an alternative method was used to generate B cell lines. Accordingly, catfish B cells were isolated by panning using mAb 9E1, a murine monoclonal antibody specific for catfish IgM [5, 8, 9]. Subsequently, surface IgM-positive cells (i.e. B cells) were stimulated with LPS and long term cultures containing only B cells were generated [8].

All the catfish LCLs grew in an anchorage-independent fashion and, with the exception of the B cell lines, contained a heterogeneous mixture of cell types. These mixed cultures have been difficult to clone, possibly due to synergistic interactions between different cell types. Recently, clonal monocyte/macrophage, B cell, and putative T cell lines were obtained by limiting dilution in the presence of 25% conditioned medium. Representative clonal lines of these three leukocyte types are depicted in Figure 1. Both clonal and heterogeneous lines divided rapidly at 27 °C, exhibited doubling times of 16–30 hr, and grew to densities greater than 5×10^6 cells/ml. Moreover, in contrast to transformed cells which are typically aneuploid, all tested lines of catfish LCLs had normal diploid chromosome numbers, i.e. 58 chromosomes.

Characterization of catfish LCLs. Catfish cell lines were classified based on previously determined characteristics of catfish B cells, T cells, and monocyte/macrophages. We defined monocyte/macrophages as nonspecific esterase-positive cells that were phagocytic and plastic adherent [3, 11]. B cells were defined initially by surface and/or cytoplasmic reactivity with mAb 9E1 [5, 9]. Further characterization of clonal B cell lines showed rearrangement of immunoglobulin heavy chain loci and the synthesis of immunoglobulin [8]. Due to the lack of monoclonal antibodies that unequivocally identified catfish



T cells, this population was defined in a negative fashion as non B cells (i.e. 9E1 non-reactive), monocytes/macrophages (i.e. nonadherent, nonspecific esterase-negative), or granulocytes (i.e. Sudan B black-negative) [3, 5].

Utility of catfish LCLs. Long-term cultures of catfish leukocytes have aided and will continue to facilitate a variety of immunological and virological studies. Moreover, it is possible to explore key immunological questions concerning immune recognition using an autologous system composed of a particular LCL and PBLs from the donor fish. For example monocyte/macrophage lines have been used to demonstrate antigen presentation and provide evidence for the involvement of MHC-like molecules in immune recognition [11–15]. Catfish LCLs have also been used to address the fine specificity of immune recognition [14–16], an issue with important implications for vaccine development. Catfish LCLs are currently being used in studies to address the role of cytokines in fish immunobiology as well as the cellular and molecular basis of cytotoxic immune responses directed against both allogenic and virus-infected cells. In addition to basic immunology studies, catfish LCLs can also be valuable resources in defining intracellular events regulating productive and latent viral infections. Finally, the ability to isolate clonal B cell lines suggests the possibility of producing fish monoclonal antibodies by generating B cells lines from immune animals. We anticipate that long-term LCLs will provide a unique source of homogeneous (or enriched) populations of distinct catfish lymphoid cells which will be valuable and unique tools to address many important questions concerning the function, physiology, and molecular biology of fish leukocyte subpopulations.

Acknowledgements

The work described herein has been supported by grants R37-AI-19530 from the National Institutes of Health, and No. 92-37208-8187 from the United States Department of Agriculture.

Figure 1. Modulation contrast images of representative channel catfish clonal leukocyte cell lines, × 300. (**A**) catfish monocyte/macrophage cell line designated 42TA-3; (**B**) catfish T cell line designated F13L-3; (**C**) catfish B cell line designated 1B10.

Notes on suppliers

1. Forma Scientific, Marietta, OH, USA
2. International Equipment Company, Needham, MA, USA
3. Brinkmann Instruments, Westbury, NY, USA
4. Bellco Glass, Vineland, NJ, USA
5. DuPoint Instruments, Wilmington, DE, USA
6. Fisher Scientific, Pittsburgh, PA, USA
7. VWR Scientific, Media, PA, USA
8. Revco Scientific Inc., Asheville, NC, USA
9. Crescent Research Chemicals, Phoenix, AZ, USA
10. Sigma Chemical Company, St. Louis, MO, USA
11. SoloPak Laboratories, Franklin Park, IL, USA
12. Difco Laboratories, Detroit, MI, USA
13. GIBCO-BRL, Life Technologies Inc., Gaithersburg, MD, USA
14. Accurate Chemical and Scientific Corp., Westbury, NY, USA
15. Intergen Company, Purchase, NY, USA
16. Corning Glass Works, Corning, NY, USA
17. Becton Dickinson, Franklin Lakes, NJ, USA
18. Millipore, Bedford, MA, USA
19. Nalgene, Rochester, NY, USA

References

1. Chinchar VG, Rycyzyn MA, Clem LW, Miller NW (1993). Productive infection of channel catfish leukocytes by channel catfish virus. Virology 193: 989–992.
2. Clem LW, Moewus L, Sigel MM (1961). Studies with cells from marine fish in tissue culture. Proc Soc Exp Biol Med 108: 762–766.
3. Ellsaesser CF, Miller NW, Cuchens MA, Lobb CJ, Clem LW (1985). Analysis of channel catfish peripheral blood leukocytes by bright-field microscopy and flow cytometry. Trans Am Fish Soc 114: 279–285.
4. Lin GL, Ellsaesser CF, Clem LW, Miller NW (1992). Phorbol ester/calcium ionophore activate fish leukocytes and induce longterm cultures. Dev Comp Immunol 16: 153–163.
5. Miller NW, Bly JE, Ellsaesser C, Van Ginkel F, Clem LW (1987). Phylogeny of lymphocyte heterogeneity: The identification and isolation of functionally distinct subpopulations of channel catfish leukocytes with monoclonal antibodies. Dev Comp Immunol 11: 739–747.
6. Miller NW, Clem LW (1984). Microsystem for in vitro primary and secondary immunizations of channel catfish (*Ictalurus punctatus*) luekocytes with hapten carrier conjugates. J Immunol Methods 72: 367–379.
7. Miller NW, Clem LW (1988). A culture system for mitogen-induced proliferation of channel catfish (*Ictalurus punctatus*) peripheral blood lymphocytes. J Tiss Cult Meth 11: 69–73.
8. Miller NW, Rycyzyn MA, Wilson MR, Warr GW, Naftel JP, Clem LW (1994). Development and characterization of channel catfish long term B cell lines. J Immunol 152: 2180–2189.
9. Miller NW, Sizemore RC, Clem LW (1985). Phylogeny of lymphocyte heterogeneity: The cellular requirements for in vitro antibody responses of channel catfish leukocytes. J Immunol 134: 2884–2888.
10. Nicholson BL (1989). Fish cell culture: An update. Adv Cell Cult 7: 1–18.
11. Vallejo AN, Ellsaesser CF, Miller NW, Clem LW (1991). Spontaneous development of functionally active long term monocyte-like cell lines from the channel catfish. In Vitro Cell Dev Biol 27A: 279–285.
12. Vallejo AN, Miller NW, Clem LW (1991). Phylogeny of immune recognition: Processing and presentation of structurally-defined proteins in channel catfish immune responses. Dev Immunol 1: 137–148.
13. Vallejo AN, Miller NW, Clem LW (1991). Phylogeny of immune recognition: Role of alloantigens in antigen presentation in the channel catfish immune response. Immunology 74: 165–168.
14. Vallejo AN, Miller NW, Clem LW (1992). Antigen processing and presentation in teleost immune responses. Ann Rev Fish Diseases 2: 73–89.
15. Vallejo AN, Miller NW, Harvey NE, Cuchens MA, Clem LW (1992). Cellular pathway(s) of antigen processing and presentation in fish APC: Endosomal involvement and cell-free antigen presentation. Dev Immunol 3: 51–65.
16. Vallejo AN, Miller NW, Warr GW, Gentry GA, Clem LW (1992). Phylogeny of immune recognition: Fine specificity of fish immune repertoires to cytochrome C. Dev Comp Immunol 17: 229–240.
17. Wolf K, Mann JA (1980). Poikilotherm vertebrate cell lines and viruses: A current listing for fishes. In Vitro 16: 168–179.
18. Wolf K, Quimby MC (1962). Established eurythermic line of fish cells in vitro. Science 135: 1065–1066.
19. Fryer JL, Lannan CN (1994). Three decades of fish cell culture: A current listing of cell lines derived from fishes. J Tiss Cult Meth 16: 87–94 (this issue).

Address for correspondence: Norman W. Miller, Department of Microbiology, University of Mississippi Medical Center, 2500 N State Street, Jackson, MS 39216-4505, USA
Phone: (601) 984 1719; Fax: (601) 984 1708