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# Marine Invertebrate Cell Culture: Breaking the Barriers

## Proceedings of an International Workshop, 16 June 1991, Anaheim, California

U. S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Northeast Region
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BIBLIOGRAPHIC INFORMATION

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Abstract: The workshop concerned marine invertebrate cell and tissue culture and was part of the World Congress on Cell and Tissue Culture cosponsored by the Tissue Culture Association and the Japanese Tissue Culture Association and held in Anaheim, California, during 16-2Ø June 1991. The workshop explored the possible reasons for the lack of success, despite vigorous attempts over a period of more than 5Ø years, to continuously grow lines of cells derived from tissues of marine and other invertebrate species. Long-term in vitro maintenance of tissues and primary cell cultures of some marine invertebrate species has been achieved--and used to good advantage. These marine invertebrate cells contain an extraordinary diversity of genetic information and possess other properties that can be used to good advantage for many basic and applied purposes.


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# Marine Invertebrate Cell Culture: Breaking the Barriers

## *Proceedings of an International Workshop, 16 June 1991, Anaheim, California*

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**Woods Hole, Massachusetts**

**March 1993**

## NOTE ON SPECIES NAMES

The Northeast Fisheries Science Center's policy on the use of scientific names in technical publications and reports is to follow the American Fisheries Society's (AFS) lists of common and scientific names for fishes (Robins *et al.* 1991)[a], mollusks (Turgeon *et al.* 1988)[b], and decapod crustaceans (Williams *et al.* 1989)[c]. This policy applies to all issues of the *NOAA Technical Memorandum NMFS-F/NEC* series.

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[a] Robins, C.R. (chair); Bailey, R.M.; Bond, C.E.; Brooker, J.R.; Lachner, E.A.; Lea, R.N.; Scott, W.B. 1991. Common and scientific names of fishes from the United States and Canada. 5th ed. *Amer. Fish. Soc. Spec. Publ.* 20; 183 p.

[b] Turgeon, D.D. (chair); Bogan, A.E.; Coan, E.V.; Emerson, W.K.; Lyons, W.G.; Pratt, W.L.; Roper, C.F.E.; Scheltema, A.; Thompson, F.G.; Williams, J.D. 1988. Common and scientific names of aquatic invertebrates from the United States and Canada: mollusks. *Amer. Fish. Soc. Spec. Publ.* 16; 277 p.

[c] Williams, A.B. (chair); Abele, L.G.; Felder, D.L.; Hobbs, H.H., Jr.; Manning, R.B.; McLaughlin, P.A.; Pérez Farfante, I. 1989. Common and scientific names of aquatic invertebrates from the United States and Canada: decapod crustaceans. *Amer. Fish. Soc. Spec. Publ.* 17; 77 p.

# TABLE OF CONTENTS

# PREFACE[1]

These proceedings are the result of a workshop jointly convened by the Tissue Culture Association's Invertebrate Division and the Society for Invertebrate Pathology. The workshop concerned marine invertebrate cell and tissue culture and was part of the World Congress on Cell and Tissue Culture cosponsored by the Tissue Culture Association and the Japanese Tissue Culture Association and held in Anaheim, California, during 16-20 June 1991. The workshop explored the possible reasons for the lack of success, despite vigorous attempts over a period of more than 50 yr, to continuously grow lines of cells derived from tissues of marine and other invertebrate species. Long-term *in vitro* maintenance of tissues and primary cell cultures of some marine invertebrate species has been achieved--and used to good advantage![2] Nevertheless, the need to develop additional "immortal" and replicating cell culture systems for long-term *in vitro* propagation of marine invertebrate cell lines is still compelling. Development of such systems will allow for the cells to be cloned, characterized, stored, retrieved, transported, and shared with cooperating laboratories. Their use will help ensure uniformity and reproducibility of results. Furthermore, the systems will provide for consistent production of an enormous variety of unique cells from many phyla. These marine invertebrate cells contain an extraordinary diversity of genetic information and possess other properties that can be used to good advantage for many basic and applied purposes. Their usefulness in the various fields of biotechnology and genetic engineering ultimately will be staggering. It is also obvious that achieving a breakthrough in the long-term continuous culture of marine invertebrate cells would go far in helping to resolve questions in comparative pathobiology research. They would be particularly useful in a parallel way, as cell lines have been useful with homeothermic, poikilothermic vertebrate, and insect cell culture systems, for the controlled propagation and in-depth study of infectious agents (such as viruses) whose growth requirements are so fastidious that they require living host-specific cells in which to replicate.

In addition to invertebrate pathologists, a number of tissue culture experts were invited to the workshop who were successful in the propagation of difficult-to-grow cells originating from a wide array of homeothermic and poikilothermic taxa, including mammals, fish, insects, and arachnids. These experts critiqued past failures. They discussed and advised those interested and involved with marine invertebrate cell culture on how to improve their chances for success to continuously cultivate cells over several generations.

Further studies were also presented by investigators currently active in research involving marine invertebrate cell biology and related fields. These reports provided additional insights for the propagation and uses of marine invertebrate cell culture. A summary report of the workshop was also presented extemporaneously by Dr. Ronald Goodwin. His interpretations of some of the information presented by the speakers drew upon his research with growth factors and membrane physiology. Dr. Goodwin was also kind enough to furnish a copy of a report prepared for inclusion in the proceedings of the Eighth International Conference on Invertebrate and Fish Tissue Culture that was held in conjunction with the 1991 World Congress on Cell and Tissue Culture. It is included as part of the summary to this workshop. Dr. Christopher Bayne also provided some additional reflections after the meeting on the report that he presented at the workshop. His later comments are also included as part of this workshop summary.

From this workshop, it is expected that many innovative ideas will be generated and may lead to some approaches for producing long-term culture of cell lines from marine invertebrates. It is also anticipated that the workshop will produce agreements for cooperative actions calling for a worldwide multilaboratory assault aimed toward the development of cell lines from several candidate species of marine invertebrates, for example from crustaceans, mollusks, echinoderms, annelids, coelenterates, and even, perhaps, primitive chordates.[3]

In closing, we thank the National Oceanic and Atmospheric Administration's Office of Ocean Pollution and National Marine Fisheries Service (NMFS) for supporting the workshop and publication of these proceedings. Special thanks are extended to the Council of the Society for Invertebrate Pathology and to the officers of the Invertebrate

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[1] The mention of trade names does not imply endorsement by the National Marine Fisheries Service.

[2] The work of Professor S.N. Chen (National Taiwan University, Taipei, Taiwan) is particularly noteworthy in connection with his studies on marine mollusks and crustaceans. See Chen, S.N., Jong, K.J.; Kou, G.H. 1989. Cell cultures derived from tissues of penaeid shrimp, *Penaeus penicillatus*, and hard clam, *Meretrix lusoria*. In: Mitsuhashi, J., ed. Invertebrate cell system applications. Vol. II. Boca Raton, FL: CRC Press; p. 253-262. See also Professor Chen's abstract, "Results of Tissue and Cell Culture of Fish and Aquatic Invertebrates," in Appendix A to these proceedings. The abstract was submitted in response to the invitation extended to him to participate in the workshop.

[3] An effort to develop a coordinated international multilaboratory program involving cell cultures of marine mollusks has already been implemented in France. A comprehensive document that defines the objectives of the program and itemizes its activities, participating laboratories, and the personnel involved, has been published. The document contains a wealth of bibliographic and other pertinent information pertaining to descriptive accounts of individual projects and related activities with other laboratories in Europe, North America, and Asia. I thank Drs. A. Guillouzo and G. D'Orange for enlightening me about this forward-looking, ambitious, and urgently needed program. Dr. D'Orange, coordinator of the program, may be contacted at: Laboratoire de Biologie Marine, Université de Bretagne Occidentale, Faculté des Sciences, 29287 Brest.

Division of the Tissue Culture Association for their encouragement and endorsement of the workshop. Mr. Bill Momberger, Executive Director, and his staff of the Tissue Culture Association helped immeasurably by making accommodations to fulfill last-minute requests for unpredicted needs. Mrs. Jane Keller of the NMFS Oxford Cooperative Laboratory has our gratitude for her role in typing and assistance in copy editing these proceedings. Mrs. Karen Hayman, also of the NMFS Oxford Cooperative Laboratory, was particularly helpful to the workshop organizers by giving special attention to matters pertaining to correspondence and program development.

A. Rosenfield (Chair)
C. Reinisch
*Workshop Organization Committee*

# MARINE INVERTEBRATE TISSUE CULTURE RESEARCH IN JAPAN

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Although marine invertebrate tissue and cell culture research is of great importance, there have been relatively few researchers engaged in this field of study in most countries. I consider it very fortunate for us to have this opportunity to meet internationally and speak about these important issues. I would like to introduce some background about marine invertebrate tissue and cell culture research in Japan.

As you will see within the citations that accompany this report, this area of study is still young. Machii's work on mantle tissue explant culture from the pearl oyster published in 1974 opened research in marine Mollusca in Japan. In this culture system, secretion of an organic substance (a kind of abnormal pearl) caused the explant to change color from milk white to dark brown. At the same time, amino acid analysis from this secreted organic substance was also performed by Yano and Machii (1975). In 1979, Ieyama *et al.* reported mitoses in the cell cultures of gonad and mantle tissue from the Pacific oyster, *Crassostrea gigas*. Development and improvement of balanced salt solution and culture media for both marine and freshwater. Mollusca were also undertaken (Machii and Kawai 1980; Kawai *et al.* 1981; Machii *et al.* 1989). Cell culture of a midgut gland from the kuruma prawn (Crustacea) was enhanced (Machii *et al.* 1988). In this study, mitoses were observed, but subculture was not obtained. In the past few years, due to the works of Awaji and Machii (1989), Awaji *et al.* (1989), Itami *et al.* (1989), Machii and Wada (1989), and Awaji and Durand (1990), tissue and cell culture research on the pearl oyster and the prawn has made further progress.

Through the Ministry of International Trade and Industry, new and exciting research has been undertaken in collaboration with major industrial firms of Japan's Marine Biotechnology Institute (Kamaishi). The institute will attempt to develop cell cultures from 39 species of marine invertebrates (Coelentrata, Mollusca, Echinodermata, Arthropoda, *etc.*) and from other marine plants and animals. Other excellent work is now in progress by Awaji (1991) of the National Research Institute of Aquaculture. His studies concentrate on the improvement of cell dissociation and purification of dissociated cells from outer epithelium of mantle tissue in the pearl oyster.

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# MARINE INVERTEBRATE CELL CULTURE RESEARCH IN NORTH AMERICA

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## MOLLUSCAN DISEASE STUDIES

It was about 35 yr ago that several marine-oriented laboratories in North America undertook concerted efforts to develop methods for the long-term cultivation and maintenance of marine molluscan cells and tissues. Using then-existing vertebrate cell culture approaches, the major objective of these efforts was to find *in vitro* systems that would allow for the isolation and identification of cytozoic pathogenic agents suspected as the cause for the devastating mortalities of eastern oyster, *Crassostrea virginica*, populations taking place along the eastern coasts of the United States and Canada: Chesapeake Bay, Delaware Bay, Gulf of Mexico, and Canadian maritime provinces. These areas at that time were the major oyster harvesting "grounds" of North America, with Chesapeake Bay representing one of the largest commercial oyster-producing ecosystems of the world. Thus, there existed socioeconomic, cultural, political, and intellectually compelling forces to find the etiologic agent(s) responsible for the observed epizootics. Furthermore, cell culture and other *in vitro* cultivation methods were expected to be used to good advantage for conducting epizootiological studies that would provide information on the spread and distribution of aquatic animal diseases, and on the prevalence, incidence, and intensity of infection of those diseases. It would naturally follow that if these cultivation methods could be developed successfully, then other biochemical and biophysical information would be acquired that would prove useful for studying: (1) host cell and pathogen gene action; (2) disease replication, transmission, infectivity, and virulence; and (3) activation-inactivation of immune mechanisms. Of course, a major consideration of shellfish resource managers was to apply the information gained to devise disease prevention, control, and eradication measures. Today in North America, molluscan cell culture efforts continue, but *in vitro* systems for cultivation of molluscan pathogens have not yet been achieved.

## MARINE CRUSTACEAN DISEASE STUDIES

During the mid- to late 1960s, and continuing even until today, disease studies on marine crustaceans have become of equal if not of greater importance than oyster or other molluscan disease studies. This is largely due to the frequent occurrences of gill and shell lesions, discolorations, and extensive unexplained mortalities of economically valuable species of shrimps, lobsters, and crabs harvested from commercial fishing grounds along the eastern and Gulf of Mexico coasts of the United States. Furthermore, worldwide interest in the rapidly expanding field of crustacean mariculture has drawn attention to the possibility that imported animals will carry infectious agents.

Several state and Federal government agencies, academic institutions, and aquaculture industry laboratories have recognized that the impact of imported disease(s), particularly of viral, fungal, or bacterial origin, could serve as major deterrents to breeding, hatchery, and growout operations, as well as to husbanding aquaculture species. We have learned a great deal from the large body of existing literature, as well as from practical experience (and the use of common sense), of the dangers inherent in the transmission of communicable diseases by infectious pathogenic agents associated with the movement of plants and animals inhabiting terrestrial ecosystems. The pathways and mechanisms for disease transmission operative in terrestrial ecosystems act according to the same principles and apply equally to aquatic ecosystems. Hence, the need is compelling for early detection and disease control strategies in which *in vitro* cell culture systems will play an important role. As with the lack of availability of molluscan cell culture systems, little progress in North America has been made toward establishing a long-term, *in vitro*, crustacean cell culture system useful for pathogen detection or for other studies.

## NEOPLASIA IN MARINE INVERTEBRATES

High prevalences of tumors and related lesions, both internal and external, have been observed in marine animals, including invertebrates inhabiting the coastal ecosystems of North America. Again, state and Federal agencies, conservationists, the fishing industry, and the public are very concerned about these findings, particularly from the viewpoints of human health, edibility of resources, and safety, as well as from the perspective of maintaining environmental (habitat) integrity. A number of investigators have undertaken activities to develop "immortal" cell culture systems employing what appears to be neoplastic cells of mollusks (oysters and clams) as found in some feral populations. Others, in order to study and compare neoplasm initiation and promotion mechanisms among the invertebrates and higher forms, are using exposure of intact animals, tissues, or cells to various carcinogens or mutagens. Some recent studies also focus on the identification, characterization, and activities of exogenous and endogenous growth promoter particles (viruses), as well as segments of nucleic acids (genes or their precursors -- oncogenes) recognized from tumorous invertebrate tissues. Attempts are also being made to determine and compare the nature of these elements with homologous entities as found in "lower" or "higher" taxa, including humans. Although some progress has been made in understanding oncogenetic processes through these studies, development of immortal cell lines using neoplastic cells and isolates from marine invertebrates has not yet been reported; efforts along these lines are, however, continuing.

## MARINE BIOTECHNOLOGY

Within recent years, there has been a veritable explosion of human activity in the field of biotechnology. Application of the term biotechnology to a number of activities involving human intrusions into and rearrangements of biological conformations and processes does not mean necessarily a new field or discipline has been developed or that a new word has been coined. Manipulations of living organisms (particularly microbial or cellular forms) and their processes so that the outcome serves the needs of mankind have been practiced since the dawn of civilization, even before. The use of plant and animal (including microbial agents) enzyme systems, extracts, and parts to accomplish various chemical and structural conversions of organic materials is well known, as are the traditional methods of plant and animal selection for breeding and hybridization. It was advances in the knowledge and understanding of gene actions-interactions and the development of DNA manipulation technology that were and continue to be major elements responsible for progress in biotechnology or genetic engineering. Application of these biotechnological approaches allows for the creative design and control of biological-genetic components and processes that can bring about structural changes or alter cellular events that, in turn, influence metabolism, reproduction, growth, development, and behavioral characteristics (*i.e.*, phenotype).

Considerable attention has been given to the use of *in vitro* cell, tissue, and organ cultures of marine plants and fishes for biotechnological applications. On the other hand, only limited activity has been directed toward use of marine **invertebrate** cell, tissue, and organ culture for biotechnological-genetic engineering purposes--largely because long-term, *in vitro*, marine **invertebrate** cell cultures for all intents and purposes do not exist or are unavailable.

As expressed in the preface of these proceedings, the needs and potential uses of long-term marine invertebrate cell culture systems are manifold. We will hear about some of the efforts and progress being made in marine biotechnological applications during this workshop; however, it is apparent that much more could and should be done by the many marine laboratories throughout the world to achieve competency for culturing cells from the several phyla of organisms inhabiting the sea and estuaries. The possibilities for biotechnological breakthroughs to develop new products and new genetic assemblages are limitless.

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# MODELS TO ILLUSTRATE THE NEEDS, USES, AND BENEFITS OF MARINE INVERTEBRATE CELL CULTURE

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Marine invertebrates offer excellent models with which to study problems in malignant transformation and cell activation. In the accompanying talk, I will present two models--one in a marine mollusk, and the second in an echinoderm--and use these examples to illustrate the state of the field and how the culture of cells could aid greatly in the resolution of fundamental issues in cell biology.

*Mya arenaria*, the softshell, develops a leukemia. The leukemia cells are detected first in the hemolymph and then in solid tissue. Monoclonal antibodies generated to the leukemia cells recognize a 190-kD, cell-surface protein. The structure, sequence, and function of this protein, which is not detected on normal cells, are totally unknown.

In fact, we do not know if leukemia cells are transformed normal hemocytes; an experiment cannot be done directly until the amoebocytes are cultured *in vitro*. Rather, our approach has been (and will continue to be) the use of immunologic and molecular probes. For example, we will generate a cDNA library for both types of cells. By subtractive hybridization, we hope to ascertain which sequences are unique to both cell types.

In a second system, developed by R.A. Prendergast and his collaborators at Johns Hopkins University, coelomocytes from *Asterias forbesi* have been shown to contain a 39-kD protein called sea star factor (SSF). This protein has profound activity on mammalian cells. SSF prevents the division of normal (unprimed) T lymphocytes to antigenic stimulation, and inhibits antibody responses by limiting the availability of lymphokines produced by helper T cells. However, in the invertebrate, when used *in vivo* in ELVAX capsules, SSF is chemotactic, drawing amoebocytes to the chamber within 24-48 hr.

In the echinoderm model, cell culture would permit the study of cytokines and growth factor receptors. Using Prendergast's approach, the phylogeny of growth factors and their receptors could be studied--work which right now is relegated to *in vivo* research because no adequate cell culture system exists.

The list of examples is endless, and additional areas will be covered during my presentation. Concerning initiatives, I firmly believe that a high priority in this field should be the establishment of tissue culture facilities solely devoted to the technology required to culture marine invertebrate cells. Funding should be cooperative between agencies committed to work in the field and **must be long term.**

In summary, immunologists are investigating clams and sea stars to resolve fundamental questions in cell biology. As examples, we are studying adhesion, transformation, and cell activation. The advent of cell culture will greatly facilitate our research and that of many others in this explosive field of work.

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# BRIEF HISTORY OF THE DEVELOPMENT OF HUMAN DIPLOID CELL STRAINS

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Exactly 30 yr ago, Paul Moorhead and I described the finite replicative capacity of normal human fibroblasts and interpreted the phenomenon to be aging at the cell level. We showed that when human embryonic cells are grown under the most favorable conditions, aging and death are the inevitable consequences after about 50 population doublings. We called this the phase III phenomenon. In more recent times, others, unaware of our work, have coined the word apoptosis to explain the same phenomenon *in vivo*.

We showed that the death of cultured normal human cells was not due to some trivial cause involving medium components or culture conditions, but was an inherent property of the cells themselves. The observation has since been confirmed in hundreds of laboratories.

At the time our observation was made, the paramount dogma in cell biology was that the failure of cells to proliferate indefinitely *in vitro* must be attributable to errors in the"art" required to keep cells dividing forever.

In describing the human diploid cell strains, we reported that these strains have several interesting properties:

1. If derived from human embryos, cell strains undergo about 50 population doublings. The potential cell yield from 50 population doublings is about 20 million metric tons.

2. Human diploid cells undergo a number of population doublings inversely proportional to donor age. This suggested to us that the finite replicative capacity of cultured normal cells is an expression of aging at the cell level. This notion received considerable experimental support in subsequent years and became the basis for the field of cell aging that we named cytogerontology.

3. If derived from normal tissue, cell strains have the diploid karyotype, and--unlike immortal cell lines--normal cell strains are incapable of replication in suspension culture. We did not give this property a name, but it is now called anchorage dependence.

4. Human cell strains will not produce tumors when inoculated into the hamster cheek pouch or even when directly inoculated into terminal human cancer patients.

5. Human diploid cell strains can be cryogenically preserved. When, for example, our first widely distributed normal human diploid cell strain, WI-38, which we developed in 1962, is preserved at a particular doubling level and then reconstituted, the number of doublings remaining is equivalent to 50 minus the number of doublings spent prior to preservation. The cells have an extraordinary memory and "remember" at what doubling level they were preserved even after 29 yr of continuous storage in liquid nitrogen. WI-38 has been preserved longer than any other normal human or animal cell population.

As a result of this characterization, we suggested that all cultured animal and human cells be classified into three groups:

1. The primary cell culture, derived from intact tissue and undergoing no subcultivations, is Group I.

2. The cell strain which has a finite capacity to replicate, does not produce tumors when inoculated into experimental animals, has the karyology of the tissue of origin, and is anchorage dependent, is Group II.

3. The cell line which is a population of immortal cells, may produce tumors when inoculated into laboratory animals, does not have the karyology of the tissue of origin, and is usually anchorage independent, is Group III. These are transformed cells.

In respect to marine invertebrate cells that do not seem to transform, this could be a blessing in disguise. Although there are reasons why immortal marine invertebrate cells might be useful, in most cases I doubt that this would be so. My objection to the use of cell lines was stated earlier; that is, these cells are not normal. Assuming that marine invertebrate cell lines would also be abnormal, their use would be limited to research in which normal cells would not be required. Then, the question becomes: Of what value are cells that do not resemble any class of cells in the anatomy of the original donor?

But this apparent dilemma does have a solution. Assuming that some marine invertebrate cell types can be cultured, and that they behave in general like vertebrate cells, one would expect them to undergo several population doublings before they age and die, just as do vertebrate cell strains. Even if the number of population doublings that these strains undergo is as few as 20, it would result in the possibility of cryopreserving well over 1,000 ampules at the 10th population doubling, and of the contents of each reconstituted ampule yielding 1,000-fold more cells. Thus, any marine invertebrate cell strain that behaved in this way could be available for use by many laboratories and for a

significant number of studies.

If abnormal immortal cell lines are useful for some purposes, one might explore the possibility of purposefully transforming marine invertebrate cell strains by the same means used for some cultured vertebrate cells--that is, use transforming conditions such as oncogenic viruses, chemical carcinogens, and radiation. If indigenous marine invertebrate oncogenic viruses are not known, this may not be important because most viral transformations made to occur in vertebrate cells succeed when the oncogenic virus is not indigenous to the animal species whose cells are to be transformed.

# TISSUE CULTURE TECHNIQUES FOR STUDY OF TRANSEPITHELIAL TRANSPORT BY TELEOST RENAL TUBULE

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The following is a brief review of tissue culture methods considered important for maintenance of tissue-level differentiation in renal proximal tubule epithelium of winter flounder, *Pleuronectes americanus*. The focus is on procedures we have found necessary to produce fully functional winter flounder renal proximal tubule primary monolayer cultures.

The value of fewer distinctly functioning segment types in marine teleosts such as winter flounder is that once the renal tubules are separated from the surrounding hematopoietic tissue, one has a relatively clean population of proximal tubule epithelial cells and may avoid certain preparative steps designed to isolate a particular cell population. Thus, choice of animal (winter flounder) eliminates one-to-several steps in the preparation of renal proximal tubule primary cultures since the proximal epithelium dominates, and cell separation techniques such as differential or gradient centrifugation are unnecessary.

Our objective has been to maintain differentiation while reorganizing the tissue from a tubule structure to monolayer form to provide a system from which definitive information about transepithelial transport may be more conveniently extracted. Because of the intimate relationship between epithelial structure and function, the establishment of morphological polarity in culture is prerequisite for the expression of net vectorial transport by cultured monolayers.

The study of transepithelial transport by cultured epithelia necessitates the use of a removable porous culture support so that cultures can be mounted in Ussing chambers. We have used native, *i.e.*, neither denatured nor glutaraldehyde-fixed (cross-linked), collagen gels. Fixed or denatured collagen cannot be contracted by any tissue. Unlike most filter supports, collagen gels are relatively transparent, so that the course of culture development can be viewed with ease microscopically. Finally, the contractability of collagen gels has been shown to influence differentiation of cultured cells, and, most importantly, allows the cells to assume a more natural cell shape in culture.

We have found that the most gentle way of isolating and dispersing flounder renal tubule cells is by cold trypsinization. In this preparation (Dickman and Renfro 1986; modifications in Gupta and Renfro 1989), the kidneys are perfused and teased apart in modified M199. The extrarenal tissues are removed by incubating the tubules in Ca- and Mg-free balanced salt solution with 0.2% trypsin at 22°C for 45 min on an orbital shaker. Tubule fragments are separated from nontubule tissues on 20-μm Nitex nylon mesh. The epithelial cells are released from these fragments by 3 days of cold trypsinization at 5°C. The cold trypsinization method was originally used by others to prevent tryptic action while allowing trypsin to penetrate tissues thoroughly. Subsequent exposure to 37°C would then produce much faster tissue dispersion. The prolonged exposure of the poikilothermic flounder renal cells to 5°C is tolerated well, and the trypsin activity at this suboptimal temperature is very gentle. The major advantage of this method is that ample time can be provided for trypsin to penetrate the tough basement membrane surrounding each nephron while minimizing enzymatic damage to the cells. Released cells are collected through 50-μm Nitex mesh, washed, pelleted, suspended in modified M199 with 10% flounder serum, and plated to confluency on native rattail or fish skin collagen.

The preparation of rattail collagen for primary hepatocyte cultures was described by Michaelopoulos and Pitot (1975). The collagen preparation we have used for the flounder renal epithelium is very similar; however, as noted, fish skin collagen also serves as an effective matrix substratum. Rattail tendons, finely teased, are sterilized with several washes of 95% ethanol, rinsed in sterile 0.1% acetic acid, and incubated in the latter for 2 days at 10°C. In our hands, sterilization of the collagen with ultraviolet light partially denatured the collagen and usually rendered it uncontractable. Gelation of the dissolved collagen is initiated by addition of 10-fold concentrated M199 culture medium and a very basic solution of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and

NaOH to the ice-cold collagen-acetic acid solution. The final collagen concentration in this mixture is 2.75 mg/ml, and the dilution factors are such that the final concentrations of salts and other nutrients are nearly identical to culture medium. In a 35-mm culture dish, 1.5 ml of this collagen solution provides an adequate substratum for flounder renal epithelial cell attachment. It is important to note that while the collagen concentration is critical, the volume of collagen solution added (and therefore the total collagen content of the dish) is equally important. The collagen mixture must be left undisturbed for about 30 min at room temperature for complete gelation. The gels are then thoroughly rinsed and equilibrated with normal culture medium.

By the same methods described above, fish skin collagen is prepared from flounder skin which has been scraped free of scales and loosely adherent tissues, and then minced. A fundamental difference in the fish and rattail collagen is the effect of temperature on gelation. Maintenance of rattail collagen plating mixture on ice very effectively delays gelation. In contrast, the winter flounder fish skin collagen plating mixture will gel rapidly even if ice cold.

The "contraction" of collagen by the epithelium is a biphasic process. The first stage of contraction occurs at a relatively rapid rate, while the second stage is much slower. Gel collagen concentration was inversely proportional to the rate and extent of contraction and seemed to influence both the fast and slow phases. Changes in the fast phase of contraction were obvious since initiation of contraction was delayed as the gel collagen concentration was increased. In fact, in our hands, it was possible to inhibit contraction almost totally by using gels that were highly concentrated. Changes in the slow phase of contraction were apparent as well since the proportionality between collagen concentration and gel size was maintained throughout the duration of the culture period. The observation that collagen concentration influences renal epithelium contraction of collagen gels may be relevant to other cell culture systems. It is also true that the use of different cell seeding densities influences contraction.

Contractable (undenatured) collagen gel substrata permit flounder renal cells to assume a semblance of their normal columnar shape in culture. When the cells are first isolated they are sphere-shaped. On plastic, they flatten when attached. This flattened appearance persists even in confluent monolayers. Transmission electron micrographs have shown that the cells assume their normal columnar shape and tight junctions on contracted collagen.

Cells which were cultured on collagen substrata of differing contractability exhibited markedly different transepithelial electrical properties. On a totally noncontractable substratum--glutaraldehyde-fixed collagen gels--flounder renal epithelium consistently exhibited lower transepithelial electrical potentials, lower total charge transfer (short-circuit currents), and higher transepithelial electrical resistances than identical cells cultured on contractable collagen gels. Cells cultured on high-density, native collagen gels which had contracted to a negligible degree also exhibited low potentials and short-circuit currents, while transepithelial resistance was intermediate to values observed for contracted gels and fixed gels.

Whether serum is present or replaced by specific hormones and growth factors, the culture medium is important in defining the differentiated characteristics expressed. The expression of tissue-level functions is obviously profoundly affected by certain culture medium constituents. However, certain effects may be insidious in that survival may be promoted while differentiation is prevented. A striking example of this can be seen in the effect of the aminoglycoside antibiotic, streptomycin, on flounder proximal tubule primary cultures (Dickman and Renfro 1990). Streptomycin is a commonly used prophylactic in culture media. Unfortunately, it is also a fairly potent nephrotoxin (Humes *et al.* 1982). In the flounder renal system, 100 μg/ml streptomycin caused total loss of all measured transport functions. The renal monolayer remained viable and readily contracted collagen gels. Ciliary activity was still apparent at the light microscope level. Thus, the source of this toxic effect was difficult to isolate due to the long-term persistence of a viable monolayer. Ultrastructurally, an almost total loss of apical microvilla was seen. A toxic effect on transepithelial transport could be detected with as little as 2 μg/ml streptomycin present, and the cells had not recovered even 6 days after total removal of the antibiotic.

Another aspect of the culture medium which must be considered is the potential for certain nutritive constituents to induce regulatory-type modification of variably expressed transport phenomena. Thus, especially in tissues such as renal proximal tubules which are subject to regulatory controls, a particular set of culture conditions must be regarded as only one of possibly many which may support varying differentiated states.

In the case of renal proximal tubule epithelium, almost all of the culture medium constituents are potentially transportable or transformable substrates. Variations in any of these ligand levels may significantly modulate a specific transport system. We have seen several examples of the effects of altered transportable substrate concentration on transport capabilities of flounder primary monolayer cultures. The tissue responds rapidly to medium phosphate concentration. Secretory phosphate flux was stimulated by increasing the medium phosphate concentration. The response to substrate change took less than 90 min. Thus, a possible important regulation of renal phosphate excretion may be phosphate itself.

In most cases, it is not clear how changes in levels of transportable substrate in culture medium cause changes in transepithelial transport processes. Factors which should be considered include the effect of the medium substrate on cellular metabolism, the possibility that the substrate may be accumulated inside the cells and promote mediated self-exchange, the possibility that a substrate may cause minor injury and induction of the stress response, and the possibility that receptor-mediated signaling through cellular second messengers is induced by certain substrates. These are

probably not discrete responses, but the latter is potentially highly specific. Second-messenger control of differentiated tissue function can be profoundly influenced by the history of physicochemical stimuli.

The development of the flounder renal proximal tubule primary monolayer culture system (Dickman and Renfro 1986) has permitted the gathering of considerable information about the transport mechanisms and regulation of teleost proximal tubule. Unidirectional transport rates for several substances which are typically handled by proximal tubule were determined in Ussing chambers under short-circuited conditions. As expected, in the marine renal tubule, sulfate is normally secreted. Organic anion secretion and glucose reabsorption are normal for proximal nephron of almost all vertebrates and are amply expressed in this culture system.

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# TECHNIQUES FOR INSECT CELL CULTURE

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This is a brief review of procedures, concepts, and results in the establishment of cell lines from four species of insects (cotton bollworm, cabbage looper, codling moth, and cat flea), and in the maintenance of primary cultures from ant and honeybee venom glands.

## MEDIA

The chemical composition of media (amino acids, vitamins, sugars, organic acids, salt, *etc.*) is obviously of concern and much has been written about the rationale for formulating media by attempting to duplicate the hemolymph biochemically. Other characteristics such as pH, oxygen tension, osmotic pressure, and ion balance may need to be optimized. In practice, however, the decision on which medium to use is usually based on a review of the literature and then choosing media that give favorable results with similar species. When evaluating media and/or modifications, one must keep in mind that there are tremendous variations in the appearance of individual primary cultures in the same medium. This makes it imperative to use many cultures (at least six) to evaluate each new medium.

In our work with media for venom gland cell cultures, we found that conditioning the medium with an insect cell line greatly increased cell viability. Several lines from Lepidoptera and Diptera were tested, and one, IPLB-SF 1254, provided significantly better growth conditions. This suggests that for other invertebrates, it may be necessary to examine media from numerous lines. Conditioning was done by using cultures with an initial cell density of $2x10^5$ cells/ml, incubating for 72 hr, harvesting the medium, removing cells by centrifugation, adjusting pH to the original value (7.0), and sterilizing by filtration. Conditioned and unconditioned media were mixed 1:1 because this combination theoretically contains the "beneficial factors" from growing cells and added nutrients from fresh medium that might have been utilized during the conditioning process.

We have evaluated numerous materials to promote cell viability, longevity, and multiplication. Most were ineffective, but some were beneficial and I want to discuss them here because others may want to test them with other invertebrate cells.

**Epidermal growth factor (EGF):** A protein extracted from mouse submaxillary glands that induces mammalian epithelial cell proliferation and DNA synthesis. At 1.83 ng/ml, it stimulated cell migration from the explants and seemed to promote cell division.

**Fibroblast growth factor:** A protein extracted from bovine pituitary glands that stimulates DNA synthesis

in mammalian fibroblasts and cells lining small blood vessels. At 25 ng/ml, it promoted long-term venom gland cell survival.

**Insulin:** Used as an effective replacement for fetal bovine serum (FBS) in a medium for *Drosophila* cell lines, and insulin-like hormones have been detected in insect hemolymph.

**Cyclic adenosine monophosphate (cAMP):** This stimulates terminal differentiation in many cells and it maintained venom gland cell viability. In the absence of cAMP, the cells developed granular cytoplasm, lysed, and degenerated within 4-5 mo. Those kept in cAMP-supplemented media remained viable for up to 12 mo. Cultures maintained in 0.05M cAMP for 3 mo and then switched to cAMP-free medium degenerated within 1 mo.

**Isoproterenol:** A catecholamine that regulates intracellular levels of cAMP and Ca++ from the cell surface. Since cAMP was beneficial, this compound was used to elevate intracellular levels of cAMP. At $5x10^{-8}$M, it promoted formation of hollow multicellular vesicles. Other materials known to elevate cAMP levels (cholera toxin, epinephrine, *etc.*) were not effective.

**Cyclic guanosine monophosphate:** At $10^{-6}$M, this produced a very striking stimulation of cell activity (initial migration and multiplication) that was more pronounced when combinedwith cAMP.

**Buffers:** Cell culture media did not have enough buffering capacity and pH dropped from the initial value of 7.0 to 6.2-6.0 in 4 days. We evaluated numerous buffers at several concentrations: BES [N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid]; MES [2-(N-morpholino)-ethanesulfonic acid]; ACES [N-2-acetamido-2-aminoethanesulfonic acid]; and HEPES [N-2-hydroxyethylpiperazine-$N^1$-2-ethanesulfonic acid]. The 0.1M BES kept the pH constant and cells in the best morphological condition.

## PRIMARY CULTURES

Embryonic tissues were used to initiate flea cell lines as they are often the tissue of choice because cells are actively multiplying and numerous invertebrate and vertebrate lines developed from embryos.

The eggs were disinfected by immersion in 1% NaOCl+ 0.1% Tween 80 for 2 min. The wetting agent in the NaOCl is important as it permits the entire surface of eggs or complete insects to be wetted, thus ensuring that all cuticular depressions are exposed to the disinfectant. Eggs are then rinsed four times in sterile water and dried on sterile blotting paper.

Since the amount of tissue from eggs is small and there is some suggestion that tissue-medium ratio is an important consideration, we cultured the tissues in standing drops. A volume of 0.15 ml was placed in the middle of 35x10-mm Lux petri dishes, the rinsed eggs transferred to the drop (10 per drop), and the embryos pulled apart with tweezers. We often refeed the cultures after 24 hr as this promotes cell viability. After the first refeeding, we replace medium every 7-10 days. However, the best schedule for medium replacement should be experimentally determined. Refeeding is done by using Pasteur pipets drawn out to a fine point, and medium is drawn up the perimeter of the drop. This prevents tissue from being aspirated into the pipet. Fresh medium (0.1 ml) is added immediately to the cultures and they are incubated at 100% relative humidity to prevent desiccation. We have kept cells alive for up to 12 mo with this technique.

If attached cells are multiplying, at some point, the surface area under the drop must be increased to give room for more cells. When about 60-75% of the area under the drop has cells, the entire surface is covered by adding 2.0 ml medium. The culture may be "reseeded" by gently flushing medium over the attached cells which frees some of them. They attach to other areas of the dish and form colonies.

It is important to keep comparatively large fragments of embryos in the drops because the presence of 4-6 floating pieces promoted the multiplication of attached cells. Of 48 cultures, only those (4) where tissue fragments were present developed into cell lines, and, without fragments, cells did not become dense enough to subculture.

Another reason for maintaining suspended tissue fragments is that they can be a source of continuously growing cells. With embryos of the codling moth, cells did not attach. After 1 mo in culture, cells began to migrate from the interior of the suspended tissues, and many layers formed on the exterior surface of the fragments. These cells descended to the bottom of the flask and formed colonies of attached cells. Within 2 mo, the primary culture contained many suspended cells and a monolayer that was subcultured.

If tissues are to be dissociated with trypsin, we find that incubation in 0.25% trypsin for 12 hr at 5°C causes less damage than incubating at 28°C for 20-30 min. After trypsin treatment, it is pipeted off and soybean trypsin inhibitor (0.1%) added directly to the tissue. The pieces are then transferred to standing drops of media, pulled apart, and agitated with tweezers. The use of soybean trypsin inhibitor, which combines with trypsin and ties up the active sites, reduces cell damage in comparison to that observed when trypsin was rinsed off.

## SUBCULTURING

A problem frequently encountered is that cells die after subculturing even though those in the primary culture are

multiplying and in good morphological condition. The critical issue is to determine when and how to subculture. Generally, we subculture when about 75% of the surface of the primary is covered with attached cells. We have experimented with numerous enzymes (elastase, collagenase, hyaluronidase, pronase) and find that trypsin is best for retention of cell viability.

Procedure for the cell line from flea tissue:

1. Use $Ca^{++}$- and $Mg^{++}$-free Hanks' phosphate-buffered saline with additional glucose to make it iso-osmotic to the medium + 0.002% ethylenediaminetetraacetic acid (Hanks' EDTA).
2. Remove medium.
3. Add 0.25% trypsin in Hanks' EDTA and incubate 10 min.
4. Remove trypsin and add fresh medium.
5. Gently rotate dish, remove medium, and add 2.0 ml fresh again (a rinse).
6. Pipet a stream of medium over the cells to flush them off.
7. Transfer total volume of dish (2.0 ml) to a 25-$cm^2$ tissue-culture flask containing 3.0 ml medium.

In early subcultures, trypsin had a delayed detrimental effect on cells observed 24-48 hr post-treatment, so it was absolutely necessary to rinse them before transfer to the flask. After the line was growing well (at the 8th subculture) and maintained in flasks, it was not necessary to rinse off the trypsin.

Procedure for subculturing an established, firmly attached cell line:

1. Remove medium.
2. Add 5.0 ml Hanks' EDTA, swirl, and remove.
3. Add 1.2 ml 0.25% trypsin and incubate 7 min.
4. Remove trypsin, twist cap on tight, and let culture sit for 3 min.
5. Bang flask against palm of hand several times to dislodge the cells.
6. Add 5.0 ml fresh medium and swirl to obtain a cell suspension.
7. Subculture every 7 days using split ratios of 1:2.5 to 1:3.3 (2.0 ml parent into 3.0 ml fresh to 1.5 ml parent into 3.5 ml fresh). After the 20th subculture, the split ratio was adjusted to 1:6.25.

Others have released attached cells using physical methods such as flushing with a strong stream of medium, scraping, or stirring with a magnetic spin bar. We find these methods unacceptable because they damage cell membranes.

# TICK CELL CULTURE TECHNOLOGY

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Based on the pioneering work of Rehacek (1965), Varma *et al.* (1975), Yunker and Meibos (1979), Yunker *et al.* (1981), and Yunker (1987), several references describe the establishment and maintenance of tick cell cultures (Rehacek 1965; Kurtti and Büscher 1979; Pudney *et al.* 1979; Yunker and Meibos 1979; Yunker 1987). In recent years, these techniques have led to the successful isolation of several new tick cell lines. Using a modification of Leibovitz's medium, L-15B (Munderloh and Kurtti 1989), we have been able to establish cell lines from every tick species tried. However, the technology to isolate, at will, lines from specific cell types has not been developed. As is necessary for the cultivation of specialized vertebrate cells, we expect that invertebrate hormones and growth factors will be important in achieving this task.

## MEDIA

The medium found to give the best results in the culture of tick cells is Leibovitz's L15 medium, which is available from several suppliers. When supplemented by FBS, tryptose phosphate broth (TPB), and, in some reports, lactalbumin hydrolysate, bovine serum albumin (fraction V), and TC yeastolate, it supports a wide range of invertebrate cells (Table 1). Antimicrobials used are standard for tissue

culture--penicillin, streptomycin, and gentamicin. The deficiencies in basal L15 are multifactorial as it lacks glucose, amino acids, organic acids, vitamins, and trace minerals known to be important for invertebrates. Based on our knowledge of invertebrate biochemistry, we modified L15 to formulate L15B which promotes tick cell growth in low FBS concentrations and the absence of TPB (Munderloh and Kurtti 1989).

## INOCULUM FOR PRIMARY CULTURES

Lines have been isolated from embryonic and developing adult tissues. Primary cultures from embryonic tissues are easy to initiate by crushing eggs, while the adult tissues must be dissected from nymphs shortly before they molt to the imago stage.

Table 1. Selected invertebrate cell lines isolated using Leibovitz's L15 medium

| Invertebrate | Cell Line Code | Inoculum | Medium | Reference |
|---|---|---|---|---|
| Nematoda | | | | |
| *Meloidogyne incognita* | MI 8C | Egg-laying females | FBS; hemolymph, *Manduca sexta* | Manousis and Ellar (1990) |
| Arachnida | | | | |
| *Rhipicephalus appendiculatus* | TTC 243 | Developing adult tissues | FBS; TPB | Varma *et al.* (1975) |
| Insecta | | | | |
| Hemiptera | | | | |
| *Triatoma infestans* | BTC 32 | Embryonic tissues | FBS; TPB | Pudney and Lanar (1977) |
| Diptera | | | | |
| *Toxorhynchites amboinensis* | TAE 12V | Embryonic tissues | FBS;TPB | Munderloh *et al.* (1982) |

Table 2. Tick cell lines banked at the University of Minnesota, Department of Entomology

| Species | Stage | Cell Line Code | Reference |
|---|---|---|---|
| *Rhipicephalus appendiculatus* | developing adult | RA 243 | Varma *et al.* (1975) |
| *Dermacentor variabilis* | embryo | RML-15 | Yunker *et al.* (1981) |
| *Rhipicephalus appendiculatus* | embryo | RAE 25 | Kurtti *et al.* (1982) |
| *Rhipicephalus sanguineus* | embryo | RSE 8 | Kurtti and Munderloh (1982) |
| *Anocentor nitens* | embryo | ANE 58 | Kurtti *et al.* (1983) |
| *Boophilus microplus* | embryo | BME 26 | Munderloh and Kurtti (1989) |
| *Dermacentor albipictus* | embryo | DALBE 18 | Witherspoon and Kurtti, unpublished[a] |
| *Ixodes dammini* | embryo | IDE 12 | Munderloh and Kurtti, unpublished[b] |

[a] University of Minnesota, Department of Entomology, 1980 Folwell Ave., St. Paul, MN 55108.
[b] *Ibid.*

## CELL LINE CHARACTERISTICS

The cell lines banked in liquid nitrogen in our laboratory at the University of Minnesota are listed in Table 2.

Initial growth of tick cells in primary cultures and early subcultures is slow, and several weeks to months may elapse between transfers. After 1-2 yr of adaptation to *in vitro* conditions during 3-5 transfers, young lines can generally be subcultured at a split ratio of 1:3 or 1:5 every 2-4 wk. Continuous lines are transferred more frequently or at higher dilutions, and have population doubling times of 3-5 days.

Lines vary in cell morphology, but generally one type predominates. The majority of cells are diploid, but aneuploid and tetraploid cells have been observed. The isozymes isocitrate dehydrogenase, malic enzyme, phosphoglucoisomerase, and phosphoglucomutase of one line (TTC 243) have been examined (Brown and Knudson 1982). We are comparing the isozymes of several different tick cell lines by isoelectric focusing gel electrophoresis, and find lactate and malate dehydrogenases to be useful enzyme systems to distinguish between lines and confirm their species identity.

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## BREAKING THE BARRIERS TO CONTINUOUS LONG-TERM CELL PROPAGATION: FRESHWATER MOLLUSKS


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The following questions and answers reflect the perspectives I have developed in the course of efforts to establish cell lines from freshwater mollusks and, more recently in collaboration with Dr. David Barnes, cell culture systems for the helminth *Schistosoma mansoni*. They have been formulated in light of those procedures which led Eder Hansen to establish the only presently available molluscan cell line (Bge; ATCC No. CRL 1494), and of the marine

emphasis of this workshop. The questions and answers are to serve as a (sometimes provocative) basis of debate and discussion.

1. *Question:* What are the qualities desired in the target species and life-cycle stage selected as the source of primary tissues?

   *Answer:* Species should be subject to propagation in the laboratory, ensuring yields of good quantities of material, at any life-cycle stage (preferably pathogen-free). Early developmental stages (embryos/larvae) are obvious subjects that have been exploited for this purpose. If hematopoietic, gonad, or other proliferating tissues are known to be available in juvenile or adult stages, these may yield suitable material for primary cultures.

2. *Question:* How completely should primary tissues be "digested" with the aim of obtaining single-cell suspensions for primary culture?

   *Answer:* Incompletely, as cells need the "nurturing" environment provided by tissue fragments (*v.* "vesicles" of insect cell culturists). A caveat is that fragments can complicate the visual evaluation of culture quality. Hansen sieved embryos to obtain single cells and small aggregates; she liberated cells using trypsin-EDTA.

3. *Question:* Does the marine source of tissues targeted here impose identifiable problems?

   *Answer:* Unknown; however, problems establishing lines from nonarthropod invertebrates have not been restricted to marine species. The necessary high osmotic values provide the scope for a great variety of media additives and relatively high concentrations.

4. *Question:* If a mechanical process is used to achieve tissue/embryo disruption, are precautions needed to reduce the risk of damage from reactive oxygen species, lysosomal proteases, and other potentially destructive products?

   *Answer:* Unknown; however, can this be achieved with superoxide dismutase, catalase, and protease inhibitor cocktails; low temperature?

5. *Question:* In the absence of knowledge about the requirements for, and the chemical nature of, growth factors in target species, can their necessity be assumed and can their functions be met by nonspecific strategies like bypassing cell-surface receptor-ligand binding, *i.e.*, directly target second-messenger systems ("shotgun") using activators of protein kinases, calcium ionophore, *etc.*?
   *Answer:* This approach, using phorbol myristic acetate and A23187, has been used successfully to immortalize fish lymphoid cells (Miller and Clem, pers. comm.[4]).

6. *Question:* When adding precious, heterologous growth factors to primary cultures, are there benefits to be gained from: (1) enzyme pretreating the cells?; (2) doing this in protein-free media?; and (3) allowing time for regeneration of cell surface receptors?

   *Answer:* Hansen trypsinized primaries "to advantage." Insect primaries may be released from inhibition by trypsinization (Schneider, pers. comm.[5]). Serum-free media are less likely to bind up growth factors.

7. *Question:* Are suprapharmacological doses of hormones, *etc.*, justified in light of the evolutionary distances which separate the producing species from the target species?

   *Answer:* Yes.

8. *Question:* Is there a role for feeder layers?

   *Answer:* Moot, in the absence of existing, osmotically compatible cell lines. (This also precludes cell fusion as an approach to be used with marine invertebrates.)

9. *Question:* When should primaries be used for terminal (fatal) assays such as nucleic acid or protein synthesis, or for live:dead determinations?

   *Answer:* Only when success has been achieved by maintaining primaries for long periods (months?).

10. *Question:* Should nonadherent cells and aggregates be included in efforts to propagate cell lines?

    *Answer:* Yes. These may yield the cell line desired, and can later be selected or encouraged to adopt an adherent phenotype.

Other factors to consider are:

1. What are the barriers? Given that numerous investigators have successfully established primary cultures from marine mollusks, much of what is written above may seem redundant. Perhaps the questions and potential solutions we need to address should center on the

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[5] Schneider, I. Department of Entomology, Walter Reed Army Institute of Research, Washington, DC 20307.

barriers themselves, their identity, and their "location" within the cell cycle:

a. Identities and lack of necessary growth hormones? Try "bypass"? (Does new information on roles of the extracellular matrix imply that attention paid to this would be rewarding?)

b. Lack of essential nutrients? Screen for ability of nutrients to improve the appearance of primaries and delay their deterioration.

c. Preculture block to propagation? Modify preparation protocols.

d. In-culture growth inhibition? Liberate.

2. Where in the cell cycle is the block? Do new stage-specific antigens in other systems provide tools for answering this? Following are some notes from Hansen's discussion of factors and problems in development of the Bge cell line:

a. Selection of tissue source. Embryos. She tried, but did not exploit, "decontrolling" the embryos' growth by irradiation and lithium chloride prior to disrupting them.

b. Sterilization. Surface sterilization of egg masses with iodine and hyamine. Primary tissues with abundant antibiotics. But these were then washed out; there were no antibiotics in regular media, though there are in the Bge medium.

c. Cell separation. Fragments tended to reaggregate. Pressing the trypsin/EDTA-treated fragments through a 30-μm sieve yielded single cells and small clumps.

d. Selecting and testing media. For primary cultures, media were often based on hemolymph composition (sugars, amino acids, and salts). The successful medium was among the simpler formulations. Once a line was established, it was simple to screen components, and to discover the utility of lactalbumin hydrolysate. Of various vertebrate sera tested, only FBS was supportive, and lot-to-lot variability was marked. Heat inactivate; avoid hemolyzed lots. The fetal calf serum (FCS) seemed essential for good cell attachment, but otherwise deleterious; it was added and removed repeatedly for primary cultures. Once the Bge cell line was established, the medium could be modified so as to be more readily available in other laboratories.

The formulation (Hansen 1976a) is: Schneider's + FCS, galactose, lactalbumin hydrolysate, and antibiotics.

Hansen considered that early use of dilute trypsin, even without passaging, was critical to her success.

3. Other considerations gleaned from the nonmarine molluscan literature:

a. Screen your lots of FBS!

b. Do you have access to water with quality adequate for high-performance liquid chromatography?

c. Don't assume cells require FBS; try them in serum-free culture.

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# CULTURED LOBSTER OLFACTORY NEURONS: UTILITY FOR STUDYING SIGNAL TRANSDUCTION[6]

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We attempted to sustain crustacean olfactory receptor neurons in primary cell culture to provide a system for studying olfactory transduction. Olfactory receptor cells in crustaceans, as in many animals, are long, thin, bipolar neurons that are not amenable for voltage-clamping odor-activated currents. Here we report a protocol that allows olfactory receptor neurons harvested from *Panulirus argus*, the Caribbean spiny lobster, to survive in primary culture for up to 23 days. The cultured cells are more compact than their counterparts *in vivo*, yet display voltage- and odor-activated properties similar to those of lobster olfactory receptor cells *in situ*.

Cell cultures were prepared from the distal third of the lateral antennular filament (olfactory organ) of intermolt lobsters, which had previously been briefly rinsed in 10% Listerine in *Panulirus* saline. Clusters of the olfactory receptor cell somata were isolated, proteolytically treated with 0.25 mg/ml of L-cysteine (1.25 mg/ml)-activated papain for 50 min, and then mechanically dissociated and plated at a density of $12x10^4$ cells/ml (per 2-$cm^2$ well) with a 61% yield. Neurons were cultured in a modified Liebowitz L15 media supplemented with salts to mimic *Panulirus* saline, basic minimal essential vitamins, L-glutamine, low dextrose, and either 6% FCS or 10% lobster hemolymph. Only one-half of the media was changed every fourth day to allow accumulation of any required neurotrophic factor.

Neurite outgrowth and cell viability were strongly affected by the type of substrate, the presence and timing of serum supplementation, and the length of time the animals were held in captivity. The cells sprouted processes within 2 hr after plating and showed optimal survival when plated on poly-D-lysine hydrobromide-coated (molecular weight 49,300-53,000) glass coverslips (2.5-5.0 $\mu g/cm^2$) or when grown on hemolymph clots. The cells did not adhere to untreated glass coverslips or collagen or laminin substrates, and adhered--but with little neurite outgrowth--to commercial plastic tissue culture wells or dishes (Corning and Falcon #1008, respectively). The cells did not adhere uniformly to the substrate when FCS was provided immediately upon plating (*vs.* 2-12 hr later), nor when the cells were kept under room light or continually agitated. The cells survived best when maintained at saturation humidity in a modular incubator chamber (Billups-Rothenberg) inside a low-range temperature incubator at 24°C (Hotpack). The cells survived equally well between 965 and 1082 mOsm, even though the actual hemolymph osmolarity is closer to the upper limit of this range -- 1079±37.5 mOsm (average ± standard error of the mean; N = 3). The addition or omission of HEPES or nerve growth factor (NGF-7s) did not noticeably affect either longevity or neurite outgrowth. Plating density dropped by one-third within the first hours of plating, with few cells extending processes, when tissue was harvested from animals held 8 wk or longer, compared to that from animals held no more than 3 wk. Preconditioned media derived from the target organ (brain and olfactory neurophil), collected at 24- and 60-hr intervals, had no obvious effect on either longevity or neurite outgrowth when used to supplement up to 50% of the culture medium.

Five morphologically distinct types of "neuron-like" cells could be defined based on their number and type of process. The somata ranged from 8 to 16 μm in diameter. Processes, when present, were less than 160-μm long. Cells of all five types were electrically excitable (N of all types = 50); voltage elicited inward and outward macroscopic currents that activated concurrently at approximately -30 mV. Stepping the cells in increments of 10mV from -60 (rest) to +30 mV activated a maximum transient inward current of 332.3±27.9 pA. The same protocol activated a maximum sustained outward current of 756.9±30.0 pA. The cultured olfactory neurons displayed odor-activated properties that were not significantly different from those of receptor cells *in situ*. Odors evoked currents of opposite polarity, and could do so in the same cell. Odor-evoked currents of both polarities were associated with a decrease in membrane resistance and a corresponding decrease in the charging time of the membrane. The current magnitudes of both polarities were dose-dependent, with thresholds as low as $10^{-8}$ M, the lowest concentration tested. The magnitude of the inward current (39.2±3.0 pA, N = 56) was significantly greater than that of the outward current (20.1±1.4 pA, N = 49; $t^1$ statistic, $p \leq 0.05$). A sample population of cells (N = 53) presented sequentially with the same five odors varied in their degree of tuning. Some cells were narrowly tuned, responding to only one or two of the test odors, while other cells were broadly tuned, responding to up to four test odors. No cell responded to the entire complement of test odors.

The latency to the onset of the outward and inward current was consistent with second-messenger mediation

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(81.0±10.6 and 186.2±31.3 msec, respectively), so we investigated this possibility. Pharmacological experiments implicated guanosine triphosphate (GTP)-binding proteins (G-proteins) in the coupling of cell-surface receptors to the intracellular effectors that produce the currents of both polarities. Including GTPγS (a nonhydrolyzable analog of GTP) in the patch pipette increased odor-evoked currents of both polarities over control (same cell without analog) by 244.2±90.0% in 8 of 10 cells tested. Including GDPβS (a nonhydrolyzable analog of guanosine diphosphate) in the pipette decreased odor-evoked currents of both polarities (N= 9) by 50.0±10.0%. Thirty-hour incubation with 1-μm pertussis toxin or 50-μm cholera toxin had no significant effect on the odor response, suggesting that the G-proteins associated with lobster olfactory transduction are bacterial-toxin insensitive.

Pharmacological experiments implicated a phospholipid second messenger in the excitatory transduction cascade. When $2.4 \times 10^{-5}$M inositol 1,4,5 triphosphate ($IP_3$) was introduced into the cultured cell through the patch pipette, 5 of 16 cells produced a sustained inward current (range of 20-73 pA) from a holding potential of -60 mV. In four cells that could be double patched, first creating a seal without $IP_3$ in the pipette (control) and then creating a second seal with $IP_3$ included (test), $IP_3$ increased the odor-evoked inward current 336.2±113.2% over that of the control. Ruthenium red (10 μM), an $IP_3$-regulated $Ca^{++}$ channel blocker, attenuated the $IP_3$-induced increase in the odor-evoked current in three out of the four cells.

These types of findings support our initial contention that culturing the olfactory receptor neurons should allow us to investigate transduction-related questions that are technically difficult to address in the cells *in situ*. For example, concern that membrane impermeant probes (*e.g.*, those targeted against $IP_3$) that, *in situ*, must be introduced through the patch pipette into the soma may not diffuse to their suspected site of action in the outer dendrites, is minimized in the morphologically compact cultured cells. The cultured cells that lack processes, but still respond to odors, presumably insert the appropriate channels into the soma membrane, which can be readily patched. Indeed, work in progress indicates this to be the case. The cultured system, therefore, appears to be a fruitful model for studying signal transduction in olfaction.

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# MARINE SPONGE CELL CULTURE


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More marine natural products have been derived from sponges than any other invertebrate group. Research on compounds with potential therapeutic application often requires a greater amount than can be supplied by collection of the organisms. The objective of our research is to develop sponge cell culture methods as an alternative to large-scale collections for bulk production of potential therapeutic agents derived from marine natural products.

A locally available sponge was selected as a model system, and a procedure was developed for initial dissociation of tissue, attachment of cells, and control of contamination. Primary cultures are initiated from both adult and larval sponges. The adult culture inoculum consists of a monodisperse suspension. The larval cultures may be started from similarly dispersed larval cells or from intact larvae. Both types of cultures may produce functional sponges.

The cultures are not axenic, and quantification of attachment and growth has been difficult. The presence of contaminating cells and the heterogeneous nature of the sponge cells themselves have made automated counting

methods unreliable. A precise quantification method is needed due to the slow and currently unpredictable responses of the sponge cells. Chromatographic techniques are used to quantify the presence and production of secondary metabolites.

While some sponges from a variety of habitats seem amenable to the same culture methods, many require adjustments at each step of the procedure. Each species presents its own challenges in terms of contaminants, cell diversity, and cell size.

Current research efforts focus on culture optimization for long-term maintenance, growth, and/or metabolite production. Though the problems of controlling contamination and sustaining cultures in a variety of media have largely been solved, some central problems remain. These include the role of microbial symbionts, the tendency of some cells to terminally differentiate, slow growth, and the presently limited life span of the cultures.

# IMPEDIMENTS TO THE CULTURE OF NONFIBROBLASTOID CELLS FROM INSECTS AND OTHER INVERTEBRATES, AND THEIR RESOLUTION

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## SUMMARY

Few demonstrable nonfibroblastoid cell types have been cultured from any eukaryotic invertebrate tissues. Most insect cell lines demonstrate no traceable differentiated functions or consistent morphologies. Outside the Insecta, primary cell or organ culture maintenance has rarely given subcultures of any cell type and almost no finite or continuous cell lines. The lack of progress in this research may be related to an inappropriate comparison of invertebrate cell culture requirements with the culture requirements of transformed vertebrate cells.

## INTRODUCTION

The fact that some insect cell types can be continuously cultured in certain vertebrate cell culture media has supported the notion that invertebrate cellular nutritional requirements are not basically different from vertebrate cellular requirements. However, the general continued failure to establish cell lines from other invertebrate groups suggests that we really do not know very much about invertebrate cell physiology. While we have inadequate criteria at present to decide whether existing insect cell lines are transformed or not (Hansen 1976), it is only cellular transformation that has permitted the isolation of the majority of vertebrate cell lines. The scientific literature describes rather startling differences between the response of transformed and normal vertebrate cells in culture to specific supplementations, particularly in serum-free media, which have been increasingly recognized as a basic requirement for nontransformed cell types. The hormonal milieu of serum has been shown to be growth preventing for vertebrate epithelial cells as well as other types, and this relationship may hold as well for many invertebrate cell types (Goodwin 1991). The complex and fastidious nature of normal eukaryotic cells in culture prevents us from extrapolating the *in vivo* concept of essential nutrients to the situation presented by these cells, tissues, and organs without significant modifications. The problems in culturing unusual (nonfibroblastoid) invertebrate cell types should, therefore, be measured against similar problems now coming to light in the culture of nontransformed vertebrate cells.

## DISCUSSION

Normal cellular growth or differentiation requirements are controlled in part through intracellular connections at the cell membrane and/or the extracellular matrix levels (Goodwin 1991). Cells in culture that maintain some intracellular membrane and/or matrix connections are often enabled to grow and may also respond to (hormonal or growth factor) commands to express tissue-specific functions *in vitro*. The scientific literature has provided many examples from both *in vitro* and *in vivo* experimentation of the control of membrane protein activity and pathogen invasion activities by specific plasma membrane lipids (Merrill 1989; Goodwin 1991). The orientation, positioning, number, and activity of growth factor and hormone receptors are controlled in part through their association with certain membrane sphingolipids, phospholipids, and sterols that are asymmetrically positioned in the plasma membrane and other internal cytoplasmic and nuclear membranes. A number of these phospholipids have also been shown to demonstrate specific fatty acid species asymmetry in a tissue-specific manner (Goodwin 1991).

While the majority of the known insect cell culture nutritional requirements resemble those of vertebrate cells (Mitsuhashi 1989), there are apparently substantial differences between vertebrate and invertebrate lipid requirements for cells in culture (Chitwood and Lusby 1991; Goodwin 1991; Thompson *et al.* 1991). Some of the apparent differences may actually be due to the differences between transformed and nontransformed cells in culture, however (Goodwin 1991). Various lipid complexes have been formulated by researchers to supplement cultured vertebrate cells either with or without vertebrate serum supplementation (Chen *et al.* 1976; Esko *et al.* 1982; Cheesebeauf and Padieu 1984). More recently, commercially available lipid-hormone concentrates and complexes, designed for vertebrate cells, have been used successfully for the nutrient supplementation of some existing insect cell lines (Goodwin 1991).

While my own research has involved liposome construction for more specific lipid supplementations (Goodwin 1989), this has not solved problems in the development of supplementations and a medium formulation that supports the continuous culture of epithelial cell types from grasshopper embryos. Certain epithelial cells in grasshoppers make up the major fat body, a liver-like tissue that is the sole replication site for the grasshopper entomopoxviruses (Goodwin 1988). In this and some of my past lipid-related research efforts, I noticed similarities to the problems around vertebrate serum supplementations that were discussed earlier by Eder Hansen and others dealing with noninsect invertebrate cell cultures. Dr. Hansen described the extreme sensitivity displayed by her molluscan cells (from *Biomphalaria glabrata* embryos) to changes in vertebrate serum supplementation evident when she changed serum lots or used different serum sources (Hansen 1976a). It was only through the careful testing of various vertebrate serum sources that she was able eventually to isolate the only molluscan cell line available to date (Hansen 1979). Since the lipid content of animal serum is highly variable and serum lipid oxidation generates toxic products, it may be that her cells were responding to serum lipid variations. Also, her work with schistosomes in her *B. glabrata* cell cultures also indicated lipid oxidation problems, since snail hemolymph supplementation was successful only when the hemolymph was pretreated with (0.1 mM) dithiothreitol, a strong reducing agent (Hansen 1976b). Likewise, the serum source was considered to be the "most important factor" in the support of molting and growth of filarial nematodes *in vitro* (Devaney 1985). And only one serum batch was effective in supporting the development of third-stage larval filarial nematodes to adults *in vitro* (Franke and Weinstein 1983). Similar serum problems led to the incorporation of a separate sterol concentrate supplementation for the improvement of tick cell cultures (Munderloh and Kurtti 1989). In my opinion, these problems all strongly suggest the need for special attention to probable specific cell-culture-essential lipid requirements.

Another problem preventing the long-term or continuous subculturing of grasshopper embryonic epithelial cells was my inappropriate dependence upon phosphatidylcholine-based phospholipids for epithelial cell culture (Goodwin 1989). It is now becoming evident that, in addition to the powerful inhibitory influence of transforming growth factor- beta (TGF-B) in vertebrate sera (Goodwin 1991; Moses *et al.* 1991), epithelial cells (and other cell types) are sensitive to the ratio of various phospholipids present, and in particular to the phosphatidylcholine-phosphatidylethanolamine ratio (Kano-Sueoka *et al.* 1990; Sutton *et al.* 1991).

A more obvious impediment to the cell culture of various invertebrate groups, as well as the culture of nonfibroblastoid invertebrate cell types, is our lack of understanding at the cell level of hormone and growth factor activity. The juvenile and molting hormones (ecdysteroids) have been widely investigated *in vivo*, but less so in cell culture. The ecdysteroids are comparable in some respects to vertebrate steroid hormones, and seem to overlay the network of more locally active peptide factors, lectins, proteoglycans, and other extracellular matrix agents, prostaglandins, and various specialized lipidic molecules. While a number of specific invertebrate hormones have been characterized (Hoffman and Porchet 1984), invertebrate cell growth factors are poorly known. It is most fascinating that homologues of EGF (Thomas *et al.* 1991) and TGF-B (Gelbart 1991) have been recognized in a number of invertebrates extending down as far as the Protozoa (Kaji *et al.* 1991) and viruses (Goodwin 1991). These factors have been compared to their vertebrate counterparts (Massague 1990), and their interactions in imaginal discs have been explored (Bryant and Schmidt 1990). It is heartening that the functional roles of these growth-factor homologues also are being revealed so quickly (Hoffmann 1989, 1990; Fehon *et al.* 1991).

Other newly described cell-active factors include an invertebrate laminin, active in the sprouting of neurons, which is not cross-reactive with vertebrate laminin (Chiquet *et al.* 1988). This factor may be related to the nerve growth factor/nerve regrowth activity described recently from a snail (Ridgeway *et al.* 1991). Now that the axiom that nerve growth does not occur in adult vertebrate animals has been contradicted by extensive experimental evidence (Nottebohm 1985; Nottebohm *et al.* 1990), it seems more likely that invertebrate cell culture systems could help reveal the growth control mechanisms for nerve cells in general.

The sequence of cell differentiation (*i.e.*, neurite sprouting) after growth and division may not be a fixed unidirectional chain of events. Apparently, eukaryotic cellular differentiation can be reversed and requires continuous regulation for maintenance (Blau and Baltimore 1991). Since the cellular oxidation state is a major influence controlling differentiation (Allen 1991), it follows that cellular lipid composition and activation, and therefore the supply of essential cellular lipids, will be critical to the maintenance or reversal of differentiation. Proof of such relationships may be slow to surface, but already the reversible activity of

cholesterol and apolipoprotein in the up-and-down regulation of gap junctions is becoming apparent (Meyer *et al.* 1990, 1991). Such activity is lipid concentration dependent and involves complex molecular interactions at the membrane level (Malewicz *et al.* 1990). This gap junction research also confirms the interdependence of lipids and proteins in cell culture systems.

## CONCLUSIONS

Recent work with invertebrate cell culture systems indicates cell surface (lipidic and extracellular matrix) specificities which significantly differ from a number of the extant vertebrate cell culture system requirements (Goodwin 1988, 1989, 1991). At the same time, significant genetic homologies are being revealed between vertebrate and invertebrate bioactive peptides. These compounds have been most useful in revealing the metabolic and functional controls of normal as opposed to transformed vertebrate cells in culture. In searching for new successful invertebrate cell culturing systems, we should be guided by a careful comparison with results published on normal stem cells and differentiation states rather than by transformed cell culture requirements.

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# BREAKING THE BARRIERS TO CONTINUOUS PROPAGATION OF MOLLUSCAN CELLS: A SUMMARY

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Let me share with you the status of research we have been doing with Dr. David Barnes and colleagues at Oregon State University in attempts to develop cell culture systems for *Schistosoma mansoni*. One year after beginning this project, it is possible to obtain cultures routinely with a variety of cell types and tissue fragments derived from schistosomules and sporocysts. The cultures remain viable for several weeks, but after the first week or so they invariably begin deteriorating. Cells that initially are distributed over the culture surface come together in groups under some conditions, and such groups appear to be potential sites for cell division. However, no cultures have yet yielded evidence of sustainable propagation.

On several occasions we refer to the protocols used by Dr. Eder Hansen in her successful attempts to establish a cell line from *Biomphalaria glabrata*, a tropical freshwater snail. Hansen used embryos as the source of her cells for primary cultures. She used high concentrations of a number of antibiotics to achieve sterile material for primary cultures. Cells were liberated by use of trypsin-EDTA and nylon mesh screens. Once healthy-looking, primary cultures were subjected to periods with and without FBS, and cultures were trypsinized without transfer to new culture containers.

A number of questions were addressed in the handout prepared for these proceedings. These questions included the qualities desired in the target species and life-cycle stage selected as a source of cells, and considerations such as how completely one should digest primary fragments to yield cells for culture. The marine sources of tissues for culture by those in the workshop should not present any particular problem; the reticence of nonarthropod invertebrate cells to propagate in culture has not been restricted to marine species. Also, the high osmolar values desired in media allow scope for the inclusion of a great variety of media components (not necessarily a good thing!) and the possibility of providing healthy levels of nutrients. This consideration may appear more reasonable in light of the very low osmolar values sometimes forced on those trying to culture cells and tissues from freshwater species, some of which have blood osmolar values as low as 100 mOsm.

It was pointed out that mechanical disruption of embryos and other sources of cells for primary culture may

induce the formation of reactive oxygen intermediates (ROI), and release proteolytic and other enzymes capable of harming cells. Precautions can include not only protease inhibitors (quite widely used), but also scavengers (such as superoxide dismutase and catalase), for ROI.

The question was raised about the possible usefulness of attempts to trigger second-messenger systems directly that are normally triggered by receptor-ligand interactions with growth factors. For example, phorbol esters and calcium ionophores might be so used. The report also appealed for retention in cultures of fragments, nonadherent cells, and anything else alive even when the morphological characteristics of such items are not those desired in a cell line; anything that grows should be accepted, and characteristics such as adherent phenotype can be manipulated or selected at a later time.

Finally, it should be noted that, in general, we do not even know where in the cell cycle that blocks to propagation arise in marine invertebrate cells *in vitro*. Are blocks to propagation inadvertently introduced in preculture protocols? Perhaps more fundamentally, we seldom, if ever, know what the putative blocks are. Could they be: (1) lack of essential growth factors?; (2) occupation of receptors by nonstimulatory analogues of growth factors?; (3) lack of essential nutrients?; and/or (4) presence of growth inhibitors? Systems need to be developed to rule in or out systematically each of these and other potential blocks to cell propagation.

# APPENDIX A

## ABSTRACT: RESULTS OF TISSUE AND CELL CULTURE OF FISH AND AQUATIC INVERTEBRATES

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Results on tissue and cell culture of fish and aquatic invertebrates have been obtained by the laboratory of S.N. Chen in the past 11 yr.

Twenty cell lines derived from warmwater fish have been established from eel (*Anguilla japonica*), tilapia hybrid (*Serotherodon mossambicus x S. niloticus*), top-minnow (*Gambusia patruelis*), color carp (*Cyprinus carpio*), common carp (*C. carpio*), snakehead (*Channa maculata*), loach (*Misgurnus anguillicaudatus*), and banded grouper (*Epinephelus awoara*), Jarbua fish (*Therapan jarbua*), striped mullet (*Mugil cephalus*), milkfish (*Chanos chanos*), and black carp (*Mylopharyngodon piceus*). These cell lines have been widely and successfully used for the study of virology, cell physiology, and molecular biology.

Attempts on the establishment of cell culture systems from mollusks have also been carried out. The results showed that monolayer cultures with approximately 10 passages have been obtained from heart, mantle, and embryos of oysters, *Crassostrea gigas* and *Ostrea edulis*, and hard clams, *Metrix lusoria* or *M. metrix*.

Monolayer cultures were also established from heart, ovary, and lymphoid tissue peripheral hemocytes of penaeid shrimp, including *Penaeus monodon*, *P. japonicus*, and *P. penicillatus*. Culture system derived from lymphoid tissue "Oka organ" was found to be susceptible to Monodon baculovirus *in vitro*.

# APPENDIX B

# ACRONYMS

| | | |
|---|---|---|
| ACES | = | N-2-acetamido-2-aminoethanesulfonic acid |
| BES | = | N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid |
| cAMP | = | cyclic adenosine monophosphate |
| EDTA | = | ethylenediaminetetraacetic acid |
| EGF | = | epidermal growth factor |
| FBS | = | fetal bovine serum |
| FCS | = | fetal calf serum |
| G-proteins | = | GTP-binding proteins |
| GTP | = | guanosine triphosphate |
| HEPES | = | N-2-hydroxyethylpiperazine-$N^1$-2-ethanesulfonic acid |
| $IP_3$ | = | inositol triphosphate |
| MES | = | 2-(N-morpholino)-ethanesulfonic acid |
| ROI | = | reactive oxygen intermediates |
| SSF | = | sea star factor |
| TGF-B | = | transforming growth factor - beta |
| TPB | = | tryptose phosphate broth |

# APPENDIX C

## WORKSHOP AGENDA

| Topic | Speaker |
|---|---|
| Introduction: Historical Perspectives | A. Machii and A. Rosenfield (Conveners) |
| Needs, Uses, and Benefits | C. Reinisch |
| Breaking the Barriers to Continuous Long-term Cell Propagation | A. Machii (Moderator) |
| Homeothermic Cell Cultures | L. Hayflick |
| Poikilothermic Cell Cultures | J.L. Renfro |
| Invertebrate Cell Cultures | |
| Insects | F. Hink |
| Arachnids | T. Kurtti |
| Freshwater Mollusks | C. Bayne |
| Other Species | S.N. Chen |
| Insights and Recent Advances Toward Understanding Continuous Culture of Marine Invertebrate Cells: Open Discussion | A. Rosenfield (Moderator) |
| Crustaceans | D. Fadool |
| Sponges | S. Pomponi |
| Other Marine Invertebrates | From the audience |
| Mollusks/Comments | L. Ellis |
| Workshop Summary: Synthesis and the Future | R. Goodwin (Moderator) and C. Bayne |

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*(continued from inside front cover)*

79. **Contaminants in Sediment and Fish Tissue from Estuarine and Coastal Sites of the Northeastern United States: Data Summary for the Baseline Phase of the National Status and Trends Program Benthic Surveillance Project, 1984-1986.** By Vincent S. Zdanowicz and Donald F. Gadbois. December 1990. x + 138p., 67 figs., 21 tables. NTIS Access. No. PB92-147453/AS.

80. **Distribution of Sexually Immature Components of 10 Northwest Atlantic Groundfish Species Based on Northeast Fisheries Center Bottom Trawl Surveys, 1968-86.** By Susan E. Wigley and Wendy L. Gabriel. January 1991. iv + 17 p., 23 figs., 3 tables. NTIS Access. No. PB92-101617.

81. **Status of the Fishery Resources Off the Northeastern United States for 1990.** By Conservation and Utilization Division, Northeast Fisheries Center. January 1991. iv + 130 p., 50 figs., 82 tables. NTIS Access. No. PB91-213785.

82. **Response of the Habitat and Biota of the Inner New York Bight to Abatement of Sewage Sludge Dumping: Third Annual Progress Report--1989.** By Anne L. Studholme, Merton C. Ingham, and Anthony Pacheco, eds. February 1991. vi + 57 p., 74 figs., 20 tables, 1 app. NTIS Access. No. PB91-208199.

83. **Organic Contaminants in Hepatic Tissues of Lobster and Flounder at the New York Bight "12-Mile" Sewage Sludge Dumpsite: 1987-88.** By A.F.J. Draxler, Paul Hauge, and Ashok D. Deshpande. July 1991. iii + 10 p., 3 figs., 8 tables. NTIS Acccss. No. PB93-114635/AS.

84. **Trophodynamics of Select Demersal Fishes in the New York Bight.** By Frank W. Steimle and Russell Terranova. July 1991. iv + 11 p., 1 fig., 16 tables. NTIS Access. No. PB92-157999/AS.

85. **Factors Influencing Spring Distribution, Availability, and Recreational Catch of Atlantic Mackerel (*Scomber scombrus*) in the Middle Atlantic and Southern New England Regions.** By William J. Overholtz, Reed S. Armstrong, David G. Mountain, and Mark Terceiro. August 1991. iii + 13 p., 9 figs., 3 tables. NTIS Access. No. PB92-160209.

86. **Status of Fishery Resources off the Northeastern United States for 1991.** By Conservation & Utilization Division, Northeast Fisheries Science Center. September 1991. iii + 132 p., 55 figs., 72 tables. NTIS Access. No. PB92-113786.

87. **Evidence of Structural Change in Preferences for Seafood.** By Steven F. Edwards. January 1992. iii + 12 p., 3 figs., 1 table. NTIS Access. No. PB93-114650/AS.

88. **Synopsis of Principal Diseases of the Blue Crab, *Callinectes sapidus*.** By Gretchen A. Messick and Carl J. Sindermann. January 1992. iii + 24 p., 13 figs., 2 tables. NTIS Access. No. PB92-219757.

89. **Proceedings of the NEFC/ASMFC Summer Flounder, *Paralichthys dentatus*, Aging Workshop, 11-13 June 1990, Northeast Fisheries Center, Woods Hole, Mass.** By Frank P. Almeida, Raoul E. Castaneda, Roman Jesien, Richard E. Greenfield, and John M. Burnett. January 1992. iii + 7 p., 8 figs., 2 tables. NTIS Access. No. PB93-114643/AS.

90. **Fish and Megainvertebrates Collected in the New York Bight Apex during the 12-Mile Dumpsite Recovery Study, July 1986-September 1989.** By Stuart J. Wilk, Robert A. Pikanowski, Anthony L. Pacheco, Donald G. McMillan, Beth A. Phelan, and Linda L. Stehlik. October 1992. iv + 78p., 9 figs., 2 tables, 2 app. NTIS Access. No. PB93-158772.

91. **The Large Marine Ecosystem (LME) Concept and Its Application to Regional Marine Resource Management** - - 1-6 October 1990, Monaco: Conference Summary and Recommendations. By Kenneth Sherman and Thomas L. Laughlin, eds. October 1992. v+37p., 3 app. NTIS Access. No. PB93-185965.

92. **Report of the Meeting of the *ad hoc* Committee on Large Marine Ecosystems, 22-23 March 1991, UNESCO Headquarters, Paris, France.** By Kenneth Sherman and Thomas L. Laughlin, eds. October 1992. iii + 19 p., 1 fig., 4 app.

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THIRD CLASS MAIL

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