BIOTECHNOLOGY IN THE STUDY OF MOLLUSCS

*Christopher J. Bayne**

ABSTRACT

The optimization of conditions and media for the *in vitro* culture of molluscan cells have yielded advances such that primary cultures can now be maintained for weeks or months. With one exception, molluscan cells have proven refractory to the establishment of continous cell lines. Knowledge derived from these studies has helped investigators maintain and grow intramolluscan parasites *in vitro*.

Recent advances in cell fusion, cell cloning and transfection of oncogenes have yet to be applied in molluscan cell culture. Once established, future cell lines will permit a great variety of studies including the control of gene expression, post-transcriptional processing including glycosylation, and the production in quantity of molluscan factors such as lectins, hormoned, immunogenic macromolecules and others of interest. In addition these lines will also facilitate screening for molluscan viruses and other cellular pathogens, and the propagation of these putative agents of biological control. Such cell lines would also permit the study of molecular mechanisms of drug and hormone actions, and the rational design of drugs.

Molecular genetic protocols have been restricted so far to studies of evolution and systematics within molluscan taxa. Both RNA and DNA probes are being developed from ribosomal, mitochondrial and nuclear compartments. Both direct sequencing and restriction length polymorphisms detected using endonucleases are yielding useful data.

INTRODUCTION

The *Chinese Journal of Biotechnology*, in a recent flyer, announces that it will publish papers in the following areas : Genetic engineering, Cell biology, Monoclonal antibodies, Fermentation, Enzymatic and bio-chemical engineering, Bioreactors; Process development and control; Automation and development of (bio-)sensors. This list provides one perspective on what should be included under the umbrella of biotechno-logy. In this review, I will focus on cell biology, although I will examine the extent to which molecular genetic approaches are beginning to contribute to our knowledge of molluscan evolution. My decision to focus on cell biology - and *in vitro* cell culture systems in particular - stems from both my greater experience with this field, and from the growing realization that advances in genetic engineering may be dependent, eventually, on the availability of cell lines in which to express genes isolated from parasites, vectors and their model organisms.

* Department of Zoology, Oregon State University, Corvallis, Oregon 97331-2914, USA

Molluscan Cell Culture

The present

In 1973, the U.S. National Institute of Health contracted with four research groups in the United States to attempt to establish cell lines from intermediate hosts of schistosomiasis. These investigators based their research on a quite extensive series of earlier studies [1]. After two years, Dr. Eder Hansen succeeded with embryonic cells from *Biomphalaria glabrata*, and the Bge cell line was established[2]. This was later characterized[3], and deposited at the American Type Culture Collection (A.T.C.C.) where it is kept in frozen storage, and is available (as CRL 1494) for use by new investigators. The cell line has been used very little, due in part to its undesirable growth characteristics (the cells tend to form aggregates, and neither form good monolayers nor grow well in suspension culture), and in part due to its evident need for very special bovine serum supplements. No further molluscan cell lines have been established, and the only non-insect invertebrate lines presently in the A.T.C.C. are derived from ticks.

Further research directed at establishing new molluscan cell lines is now desirable. The variety of uses to which such lines may be put has expanded as molecular biology has grown. Furthermore, new knowledge of what it takes to encourage at least vertebrate cell population growth *in vitro* has placed new possibilities before us.

The future of molluscan cell culture

Sources of cells. Most of the molluscs which transmit parasites to humans and (our) livestock are gastropods, and these should be the organisms of choice for future efforts to establish cell lines. For several species, protocols and media already exist for prolonged maintenance of primary cell cultures in good condition[1]. The best species from which to grow cells will be those which can be cultured easily and inexpensively in captivity. This is desirable because, unlike field-collected specimens, lab-reared individuals can be obtained free of parasitic infection. The species should be those that reproduce prolifically, since embryonic material is likely to be optimum for cells with good growth potential, and the investigators need to have access to large quantities of material.

Hygiene. Since the inclusion of antibiotics in culture media may interfere with growth of the molluscan cells, alternatives should be sought. Surface sterilization of the donor animals may be complicated by the tendency of molluscs to secrete surface mucus. Use has been made of developing egg masses as sources of cells[2], and disinfectants such as 0.2 % iodine, 1 % benzylkonium chloride, 70 % ethanol, 0.001 % hyamine, and 0.5 % hypochlorite have been used for surface decontamination. We hold our snails in a solution of penicillin, streptomycin and fungizone for 30 minutes to 1 hour prior to surgery, and surface sterilize the shells with 70% ethanol.

Obtaining cells for culture. Explanted tissues should be those with mitotic potential and low risk of microbial contamination; digestive glands should be avoided on account of the latter. Gonads, tissues producing blood cells (hemocytes), and embryos should be best. It is inadvisable to explant large bits of tissue. Therefore, on removal from the animal, the tissue should be minced by cutting or by being pressed through a sieve using a rubber plunger. Prior to placement in primary culture, the tissues should be treated to facilitate the emigration of cells, or to yield

single cell suspensions for primary cultures. Typically, trypsin and E.D.T.A. are used, but molluscan tissues may be better dissociated when attention is paid to the probable involvement of carbohydrates in the extracellular matrix. Thus carbohydrases, hyaluronidase and the addition of sugars[4] need to be considered. If extensive cell damage is expected, then a cocktail of protease inhibitors (such as P.M.S.F., leupeptin, pepstatin, alfa2-macroglogulin) may enhance the vitality of the remaining intact cells.

The previous solutions should be replaced as soon as possible with one containing divalent cations and protein, as this serves to further enhance cell survival. Viability should be scored by dye exclusion, such as trypan blue or propidium iodide. Preparations with viabilities less then 90% should be rejected.

Culture conditions. Among the numerous factors which will determine the subsequent fates of explanted cells, the nature of the culture surface is important. Cells with very high affinities for a culture surface may be inhibited from cytokinesis. Hemocytes, for example, with their strong reactivities to foreign surfaces, may benefit from the inclusion of agarose or methyl cellulose to the medium, to reduce their adhesiveness. Culture surfaces may be improved by "conditioning", through overnight exposure to the culture medium. Simple solutions of serum albumin or fetal calf serum may improve culture surfaces.

There will be many variables to be tried with respect to composition of the culture medium. A screening protocol which was used productively by Eder Hansen included establishing all primary cultures in one basic medium known to support primary cell cultures for at least several days (preferably weeks), then replacing this with test media using replicate cultures. As an indicator of the merits of a medium, it has been standard practice to monitor DNA synthesis by the cellular incorporation of ^{3}H-thymidine. However, new developments in biotechnology now allow for non-isotopic assays. Antibodies can be used to detect cells which have incorporated bromodeoxyuridine into their DNA (kit available from Amersham), and it can be hoped that, if the newly discovered cyclins prove to be widespread, their presence in cells will be detectable with a standard protocol which would reveal those cells which are at specific stages of the cell cycle in a population.

Patience is essential for successful establishment of cell lines. Primary cultures need to be left for weeks, with regular microscopical evaluation to determine their condition. When evidence of cell division is seen, then it may be advisable to harvest the cells, thereby disaggregating them, and allow them to re-settle on the original culture surface, without splitting the culture. This procedure may remove contact-inhibition of cell growth. As cultures approach double or triple the original cell number, they may be split. One half should be transferred to a new culture vessel, and the other should be returned to the original, to reduce the trauma of transfer.

Developments in molecular genetics have placed in our hands tools which may be useful for inducing cell population growth even in taxa distinct from those in which the tools have been isolated. Such tools include oncogenes like *ras* (Barnes, personal communication), and growth-promoting peptide hormones. It is the highly conserved nature of many hormones and their receptors that makes the use of hormones from heterologous species potentially rewarding. Cell fusion is another approach which remains to be tried with molluscan cell cultures.

Potential uses of molluscan cell lines

In order to be assured that post-transcriptional processing of molluscan genes approaches normal, the expression systems should be as close as possible phylogenetically to the species from which the gene was isolated. Clearly cell lines have potential value in this area of endeavour.

A second application of molluscan cell lines may be for the production of endogenous molecules. For example, there has been interest in the finding that molluscan intermediate host species such as *Biomphalaria glabrata* express antigens known also from their parasites, in this case *Schistosoma mansoni*[5]. The host, then, could be a source of antigens with potential use in vaccines. Large scale culture of the mollusc's cells could be developed for the production of such molecules.

A third important potential application of new molluscan cell lines is not strictly biotechnological. This is their use in screening for molluscan viruses or other cellular pathogens. There is at present not a single available or even identified microbial or viral agent of known pathogenicity to gastropod molluscs.

Novel Approaches to Cell Surface Labelling

The cost, inconvenience and safety concerns associated with the use of isotopes in the laboratory are stimulating the development of new reagents and protocols for the non-radioactive detection of molecules in trace amounts. One of these - the biotin-avidin system - has been used to examine the surface chemistry of snail hemocytes[6]. This is briefly described here, as it has great potential for research in host-parasite systems where sensitive, non-isotopic detection of macromolecules is needed.

Hemocytes present difficulties to the investigator hopeful of labelling their cell surfaces, on account of their strong tendency to clump, which removes large areas of the cell surface from access by the label. This could be overcome by exposing the cells to biotin before the cells could encounter one another. The NHS-biotin was added to a solution of 5% Ficoll in a nutrient medium, and the snail hemolymph was carefully layered on top, then immediately centrifuged. The plasma is retained above the Ficoll, but the individual hemocytes pass through it during centrifugation. During descent, the NHS-biotin labels surface- exposed proteins on the cells. It does not enter the cells, which remain viable. The medium is removed, the cells are washed with 0.1 M Tris-HCl buffer to inactivate any remaining biotin, and then SDS sample buffer is added. Boiling the preparation for 3 minutes solubilizes the macromolecules and inactivates any endogenous proteolytic enzymes. The only biotinylated peptides are those which were exposed on the surface of the living cells. Following electrophoresis, the separated components are electroblotted onto nitrocellulose where the labelled peptides are detected by means of streptavidin-alkaline phosphatase followed by a color-development reagent. This system is capable of detecting a few picograms of a peptide on the membrane.

The NHS-biotin used in this study can be replaced by biotin- hydrazide which labels carbohydrates, or by a photo-activatable derivative of biotin for the labelling of nucleic acids. The streptavidin can, of course, be labeled with fluorochromes like FITC, lectins, or alternative enzymes. This is a remarkably versatile technology. and will be used increasingly. Useful, clear protocols are

often provided free by the suppliers of reagents. In the case of the biotin-avidin system, Vector Laboratories in Burlingame, California, and Pierce in Rockford, Illinois, are useful sources of information. Very clear protocols are also given by Harlow and Lane[7].

On the Use of Antibodies and Lectins as Probes

Most of the successful uses of antibodies as probes has been with vertebrate, indeed mammalian, systems. As parasitologists and comparative immunologists adopt these reagents to probe for antigens in invertebrates, certain precautions are advisable. Invertebrates lack the lymphocytes and immunoglobulin antibodies which characterize the immune systems of all vertebrates. For the detection of non-self, therefore, they depend on different recognition systems, probably in large part based on lectin- carbohydrate interactions. This implies that carbohydrates may be critical for the integration of functional systems in these animals. Specific carbohydrate "epitopes" may be just as unique in their molecular characteristics as the more familiar peptide epitopes, but *much more widespread in occurrence* in invertebrates, at least. Carbohydrate epitopes are, of course, fully immunogenic, and mammalian antisera commonly contain antibodies to such epitopes. It follows that antibodies developed in mammals against single antigens from invertebrates or protists, if those antigens contain carbohydrate determinants shared with other macromolecules in the intact organism, will detect these other antigens in mixtures. This has been found to be the case with the plasma proteins of *Biomphalaria glabrata*[8]. A rabbit antiserum raised to a single lectin from the snail plasma recognized the majority of peptides in the whole plasma. Caution is advised when equivalent probes are applied in studies of parasites and their vectors. Talk of "homology" or "identity" of molecules on the basis of serological cross-reactivity is ill-advised unless accompanied by further, independent molecular characterization.

Nucleotide Sequences and Systematics

Phylogeny reconstruction[9] can be focussed at the level of recent speciation events or at more ancient and distinctive evolutionary divergences such as between Classes or even Phyla, or at any level in between. The choices are reflected in the nature of the genomic analyses which are used.

At their 54th Annual Meeting in 1988, the American Malacological Union convened a symposium entitled *Applications of Nucleic Acid Techniques to the Study of Molluscan Evolution*. Of the twelve papers, eight dealt with the mitochondrial genome - its size within a species (estimates of 14.5 kb, 17.3 kb and 32 kilobase pairs; Murray, B.L. Brown, Fuller), variation in mitochondrial DNA (mtDNA) sequences between congeners (Palmer, W.M. Brown, Stine, B.L. Brown), between cognate species (Collins) and between sister species (Hoeh). Another two papers reported studies on ribosomal RNA (rRNA), one of which evaluated evolutionary rates between several bivalve genera (Bowman), and the other of which included both bivalve and gastropod genera (Kuncio). Ribosomal DNA from the Asiatic clam *Corbicula*, a relatively recent immigrant to North America, was subjected to restriction endonuclease analysis in an attempt to sort out a disagreement between systematists as to whether or not two sympatric morphs represent distinct species. According to the author (Hillis), they do.

A search through the Scientific Citation Index up to March 1989 revealed that none of the studies reported in that symposium had yet been published. Nevertheless, it is clear that the powerful tools of nucleic acid sequencing, restriction fragment length polymorphism analysis and genomic probing are being used to answer questions concerned with molluscan evolution. An "avalanche" of papers in this area can be predicted with confidence.

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QUESTIONS AND ANSWERS :

1. Question: Which expression system is best for studying antigenic determinants of a given parasite/microbe ?
   (my field is in microbiology/Imunovirology) Thanks.

   Answer : If peptide is a "base" peptide. Then *E.coli* or the microbe would be fully acceptable. Dr. Loverde explained that post-translational phosphorylation, glycosylation etc would require eukaryotic expression system. For precise glycosylation-such as addition of multiple glucoses in schistosome proteins - a homologous expression system would be desirable.

2. Question: Has mutagenesis (or sublethal irradiation) of molluscan cells been attempted to initiate immortal cell lines ?

   Answer : Yes. Both UV light and chemical mutagens have been tried unsuccesfully. More work is needed (Cell division may have not occurred due to difficulties with cytokinesis).

3. Question: Could you give an information to obtain mollusc extract for enzyme electrophoretic method?

   Answer : Avoid the digestive gland due to
   1. proteolytic enzymes and carbohydroses
   2. symbionts (contaminants)
   Extract in physiological buffered saline with enzyme inhibitors, and spin out the pellet. Use supernatant right away.

4. Question: What difficulties that prevent the establishment of molluscan culture (cell line) up to now? What about the short term molluscan culture ?

   Answer : Short-term culture is potentially very useful. Primary cultures have been maintained for a year *in vitro*. Culture media are available. We do not know what is needed to establish molluscan cell lines this is where research is needed.