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Bivalve Mollusc Cell Culture

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Abstract.—The historical background of bivalve cell culture and its potential uses in molluscan pathology are reviewed. Then, primary cultures for the study of some host–pathogen interactions at the cellular level are considered. Next, alternative methods to classical cell cultures are examined that may lead to the production of bivalve cell lines.

Bivalve breeding, an economically important element of aquaculture, is becoming increasingly subject to the hazards of disease. Infectious diseases affect the species of bivalves bred throughout the world and are caused by intracellular pathogenic agents, viruses, microorganisms, or parasites. The diseases develop into epizootics that are responsible for considerable economic loss.

The study of these diseases must go beyond description to establish methods for prevention and therapy. Given the results obtained in human and veterinary pathology, the achievement of such measures depends partly on the development of bivalve cell cultures that permit in vitro cultivation of the intracellular pathogenic agents. Such cell cultures could help us to determine and understand the host–pathogen relationship at the cellular and molecular level (Trager 1983; Agabian and Eisen 1985). Practically, with the attainment of in vitro production of parasites, experimental reproduction of the disease could lead to the selection of resistant animals. The cultures would also constitute a diagnostic tool adapted to revealing fastidious pathogens such as viruses (Fields 1985). In addition, cell cultures would permit in vitro screening of chemical substances as candidate antiparasitic agents (Campbell and Rew 1986).

Cell cultures of marine molluscs date from 1960s and were inspired by the establishment of several insect cell lines (Vago 1972). In these cultures of molluscs, bivalve tissues were maintained in simple culture media to which vertebrate serum was added (Chardonnet and Peres 1963; Tripp 1963; Perkins and Menzel 1964; Li et al. 1966; Tripp et al. 1966; Vago 1972). The cells survived only a few days or a few weeks in spite of claims that cell lines had been established (Vago and Chastang 1960; Doutko 1967).

More recently, as a result of several economically catastrophic viral and parasitic epizootics (Sindermann 1976; Meuriot and Grizel 1984; Grizel 1985), research on the culture of bivalve cells was resumed (Cousserans 1975; Brewster and Nicholson 1979; Stephens and Hetrick 1979a, 1979b; Hetrick et al. 1981). These experiments gave rise to improved techniques for the establishment of primary cultures, especially of embryonic and larval cells, but cell lines were not established. This failure underscored certain difficulties: the need for practical methods of regularly obtaining aseptic tissues, which are essential for the attainment of primary cultures (Millar and Scott 1967), and the lack of physiological and biochemical data needed to devise suitable culture media.

Nonetheless, the establishment of an embryonic cell line of the bloodfluke planorb *Biomphalaria glabrata* (Hansen 1976; Bayne et al. 1978) supported the feasibility of experiments in cell cultures of bivalves. This success and the understanding that cell systems are essential for progress in bivalve pathology indicate the need for further research in this area. Short-term research should focus on primary cultures and their direct use for in vitro study of intracellular pathogens; longer-term work should address methods for establishing stable cell lines. These areas of research are considered in this article. The technical aspects of cell culture can be found in more specialized publications (Malek and Cheng 1974; Bayne 1976; Jakoby and Pastan 1979).

Primary Cultures

Primary cultures are those initiated either from explants (from which the cells migrate) or from cells that have been isolated by mechanical or enzymatic dissociation of tissues. Theoretically, any cell type may be placed in culture, but its survival in vitro varies greatly according to its nature and degree of differentiation.

The major difficulty in establishing primary

cultures rests at the preliminary decontamination of the tissues. The majority of bivalve organs are in direct contact with the environment, which is contaminated by various types of microorganisms and protozoans. Some relatively efficient protocols for decontamination have been developed for adult tissues (Stephens and Hetrick 1979a, 1979b). The presence of numerous intrinsic contaminants impedes the attainment of aseptic tissues from the digestive gland. For larval tissues, the most reliable method is in vitro fertilization with gametes removed aseptically.

Primary cultures should be maintained in culture media whose composition is based on the physicochemical characteristics of bivalve hemolymph. Biochemical data on the hemolymph are limited to concentrations of free amino acids (Bishop et al. 1983), total protein and sugar contents (De Zwaan 1983), and certain inorganic constituents (Deane and O'Brien 1979). These data, however, vary greatly between species, between individuals, and according to the physiological condition of each animal. On the other hand, certain physicochemical criteria, such as osmotic pressure and pH, are better defined (Burton 1983). After these characteristics are taken into account, certain components used classically in cultures of animal cells, are added to the medium. These include compounds such as nucleotide precursors, vitamins, and growth factors. Other additives may include fetal calf serum, yeast hydrolysates, and vitamin solutions. In some experiments, media have been supplemented with homologous hemolymph (Boulo, unpublished data). An important feature of molluscan cells seems to be a low nutritive requirement, because primary cultures generally show longer survival in media diluted with sterile seawater.

Cell culture work in our laboratory with the edible oyster *Ostrea edulis* leads us to the following observations.

(1) Cells taken from paleal and palpeal tissues are of two types: ciliated cells that lose their cilia after a few days of culture and then degenerate rapidly; and smaller, spherical, less-differentiated cells that remain in suspension and survive for several weeks without showing mitotic figures.

(2) Primary cultures of larval cells are distinctly more promising. In particular, cultures established from veliger larvae are at the optimal stage of differentiation. At an earlier stage, the cells are too charged with vitellus and are very fragile. At a later stage, the larval tissues are too differentiated and newly formed shells constitute a major obstacle to tissue dissociation. Mechanical dissociation of young larval tissues is preferable to enzymatic dissociation because it is less stressful for the cells. Mechanical dissociation regularly leads to a large degree of adherence of explants and much cellular migration. Cell activity remains apparent for several weeks as contractile elements cause a rythmic movement in certain explants. Nonetheless, as of the sixth week of culture, the cells progressively change and become suspended without any observable cellular multiplication.

(3) Primary cultures of hemocytes have been easy to establish because of the low risk of contamination during removal of the hemolymph and the ease with which the promial vein can be punctured. Hemocytes in culture regularly survive 6 weeks.

Thus, primary cultures already constitute cellular systems that permit a short-term approach to the culture and in vitro study of pathogenic agents. Primary cultures of embryonic and larval tissues of the Pacific oyster *Crassostrea gigas* could be used, for example, to study the iridovirus responsible for oyster velar disease (Elston and Wilkinson 1985). Such a procedure has already been used to cultivate insect viruses and rickettsias in vitro (Vago and Quiot 1982).

Primary hemocyte cultures can be easily established throughout the year and maintained for periods regularly lasting 4–6 weeks. They constitute a select system for cultivating viral or protozoan pathogens, which develop in the hemocytes of different species of bivalves. The isolation and purification of *Bonamia ostreae* (Mialhe et al. 1988) has enabled us to experimentally infect primary hemocyte cultures and undertake the in vitro study of the relationships between this protozoan and the hemocytes of both the naturally susceptible edible oyster and the experimentally resistant Pacific oyster. This model has already made possible the description of the initial stages of parasite development, and has provided an approach to the mechanisms of recognition, penetration, and survival in the hemocyte. This model is comparable to those in human and veterinary parasitology (Trager 1983) for parasites for which there is no experimental cell line (e.g., *Plasmodium* spp., which develop in erythrocytes).

Cell Lines

Subsequent to cell multiplication and at the time of the first subculture, primary culture becomes a cell line. A cell line thus corresponds to a population of cells whose capacity for multiplica-

tion is unlimited. Hence, development of an empirical and uncertain nature is the origin of numerous cell lines (Hink 1980), including the only cell line of a mollusc, the bloodfluke planorb (Hansen 1976). Generally, primary cultures are established from tumors, embryonic and larval tissues, or connective tissues. The different tissue types have a low degree of differentiation, and mitotic activity is characteristic of the tissue of origin. The principal advantage of cell lines over primary cultures lies in their capacity for multiplication. Thus, the ability to obtain a cell line from host tissue, if it is susceptible to infection with a given pathogenic agent, will greatly facilitate in vitro studies of that pathogen.

The experience acquired in working with animal cell lines confers a special a priori interest in culturing embryonic and larval cells of bivalves. This was also the choice of Hetrick et al. (1981), who used embryos of the eastern oyster *Crassostrea virginica* at the 16- and 32-cell stage. Our work with embryos of edible oysters has led us to select young veliger stages for the reasons already given. Also, larval production in a hatchery can be performed throughout the year, a major advantage for conducting continuous experimentation and for optimizing culture conditions.

The infrequent observation of mitosis in primary cultures suggests the absence of mitogenic factors in the culture medium. This underscores the need to test various supplements to the medium, such as homologous hemolymph or extracts from cerebroganglions of the common Atlantic slipper snail *Crepidula fornicata*; the latter contains a nonspecific mitotic factor (Le Gall et al. 1987). Tests could also be conducted with extracts of genital glands, which may prove favorable to cell multiplication (Anonymous 1985).

It is also important to undertake experiments in tumoral transformations, either in vivo or in vitro. Tumors occur naturally in bivalves (Mix 1986; Peters 1988, this volume), and some knowledge has been acquired about chemical carcinogenesis (Heidelberger 1975; Hecker et al. 1982; Huberman and Barr 1985; Huberman and Jones 1985). Chemical carcinogenesis is, at present, considered a two-stage process in which sequential events, initiation and promotion, lead to the appearance of tumors (Hecker et al. 1982). Initiation presumably involves an irreversible "mutational event of a tumor gene." This event is caused by a carcinogen capable of binding covalently to DNA and forming DNA adducts. Some chemicals bind directly (nitrosamides) and others only after cellular conversion to a chemically reactive form (polycyclic aromatic hydrocarbons, aromatic amines, nitrosamines, aflatoxins). The second stage of the carcinogenic process is tumor promotion (Diamond et al. 1980). Tumor promoters are devoid of mutagenic activity but enhance cell transformation. They may exert their promotional effect by causing the expression of mutated tumor genes similar to gene expression during cell differentiation. Some experiments with molluscs have been reported that involved benzo[*a*]pyrene, 3-methylcholanthrene, and a direct-acting mutagen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Hetrick et al. 1981). The negative results observed in vivo and in vitro could be explained by the absence of tumor promoters. With initiator and promoter chemicals, some positive experimental results have been reported in vivo for a planarian (Hall et al. 1986) and in vitro for human and rodent primary cultures (Huberman and Jones 1985). These experiments have stimulated our own research with edible and Pacific oysters now in progress in our laboratory.

Finally, it will be useful for molluscan pathologists concerned with cell culture to take into account research on oncogenes (Bishop 1983; Bradshaw 1986; Garrett 1986; Klein and Klein 1986), because neoplastic transformation (Ratner et al. 1985) of vertebrate primary cells was achieved by transfection with active oncogenes. Oncogenes are widely conserved evolutionarily from yeasts to insects to mammals (Shilo and Weinberg 1981; Jenkins et al. 1984).

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