Special Issue

Culture of cells from two life stages of *Schistosoma mansoni*

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Abstract

The ability to culture continuously proliferating cell lines of various organisms *in vitro* has provided numerous advantages in experimental approaches toward the understanding of basic biology and disease. Although *in vitro* approaches are common in many disciplines, this methodology has proven difficult to exploit in the study of helminthic parasites. A major cause of parasitic disease, particularly in tropical countries, is the trematode *Schistosoma mansoni*. We have developed *in vitro* techniques that allow the long term maintenance of cell cultures from two stages of the life cycle of this organism, associated with its mammalian and the molluscan hosts. We have developed quantitative assays of cell survival and proliferation in our culture systems, and obtained evidence for limited proliferation *in vitro*. Although the cultures we have achieved thus far are useful for many kinds of experiments *in vitro*, development of continuously proliferating cell lines remains our goal.

Introduction

The parasitic helminth *D. mansoni* infects hundreds of millions of humans world wide, and is a major cause of parasite-related mortality. Efforts toward control of schistosomiasis are being approached at both the basic and applied levels. It is well recognized that *in vitro* cell culture systems provide advantages for many kinds of experiments (Barnes, 1987). *In vitro* systems have long been available for cells from mammals, birds, lower vertebrates such as fish, and some invertebrates, such as insects and ticks. However, cell cultures of non-arthropod invertebrates are uncommon, and continuously proliferating cell cultures (cell lines) from such organisms are rare (Bayne, 1976; Hansen, 1976).

Methods have been developed for *in vitro* culture of intact forms of some stages of the life cycle of *S. mansoni* (Clegg, 1965; Basch, 1981; Stibbs *et al.*, 1979; Newport and Weller, 1982). However, attempts to define a cell culture system for *S. mansoni*, primarily directed to the culture of cell from the adult worm (mammalian host) stage (Weller and Wheeldon, 1982; Coles and Fitzgerald, 1986), met with limited success and were subsequently abandoned. We took a fresh look at the potential to develop cell culture systems

for *S. mansoni*. In experiments directed toward culture of cells of the mammalian life cycle stage, cultures maintained viability for up to six months (Hobbs *et al.*, 1993). In experiments directed toward the culture of cells from the intramolluscan larval stages, cells from primary sporocysts were maintained in complex media containing fetal bovine serum (FBS) and viability was sustained for several months. The predominant cells ultrastructurally were germinal cells (Bayne *et al.*, 1994). Germinal cells are the sporocyst cell type committed to rapid, undifferentiated growth in this stage of the life cycle of the parasite. To our knowledge, this work is the only attempt to culture cells from sporocysts of *S. mansoni*.

Exploration of the cell culture system will furnish information on hormonal, nutritional and other extracellular factors that may influence viability, virulence or fecundity of the organism in the host, and thereby provide a means toward improved drug design (Weller and Weldon, 1982). Cultures also might supply a dependable source of parasite-derived factors that may modulate the immune response of the host and be of significance in the pathology of the disease (Pearce and Appleton, 1992; Duvaux-Miret *et al.*, 1992). In addition, cell cultures could be used in large scale screens

of metabolically-directed drugs in an economical manner and to elucidate the biochemical and genetic basis of drug resistance by selection of resistant cells *in vitro* or by culture of cells from resistant strains of the parasite (Coles and Vruce, 1987).

Results

Culture of cells from the mammalian stage of the life cycle

Juvenile worms isolated by perfusion of livers 12–21 days after infection were disaggregated and cultured. Preparation of juvenile worm cultures involves mechanical disruption, several enzymatic digestions and serum-free plating. Particularly important is digestion with a crab-derived collagenase, which is superior to mammalian or bacterial-derived collagenase for tissue disaggregation. The schistosome interstitial matrix is largely composed of collagen-like fibers, and the crab collagenase may simply work better on this matrix than other collagenases. The crab enzyme also exhibits chymotrypsin-, trypsin-, and elastase-like activity. A beneficial effect of contaminants in the enzyme preparation is also possible.

The medium used was based on a low-phosphate Dulbecco-Modified Eagle's medium formulation (commercially available) and modified according to information concerning *in vitro* requirements of intact worms from Basch (1981), information concerning requirements of cells from adult worms from Weller and Wheelodon (1982) and George Newport (personal communications; Newport and Weller, 1982), and our own experimental observations. Additional components included orotic acid, glutathione, nucleic acid precursors, cholesteryl lineolate, selenium, and ampicillin (Hobbs *et al.*, 1993). Best results were obtained with 16–18 day-old juvenile worms. Antibiotics were not detrimental in our cultures, unlike the adult worm cell cultures of Weller and Wheelodon (1982), but in many other instances (eg., optimal concentrations of calcium and phosphate), we confirmed the previous observations of these researchers. No improvement was observed when the medium was supplemented with a variety of factors that are effective in the culture of vertebrate cells (Barnes, 1987). Other standard basal media formulations were less successful at maintaining viability. These included Schneider's *Drosophila* Medium, DME, Lebowitz's L-15, Ham's F-12, F-12: DME (Barnes, 1987), medium F (Stribbs

et al., 1979), Swim's S-77, Medium 199 and RPMI.

Cultures contained small round cells (5 μ m or less diameter) adherent to the plastic cell culture surface individually or in groups of 5–25 cells. Individual bi- or multi-polar cells were physically connected to larger clusters of cells and contracted arrhythmically. Areas of some clusters concentrated phenol red (medium pH indicator), a phenomenon previously reported in cultures derived from adult worms (Weller and Wheelodon, 1982). Viability was assessed by a double fluorescent dye staining procedure employing Hoechst 33342 (staining live and dead cells) and propidium iodide (staining dead cells) (Hobbs *et al.*, 1993).

Long term culture was accomplished by the use of a feeder layer of irradiated rat liver cells (BRL cell line). For preparation of feeder layers, BRL cells were grown to confluence in 75 cm² flasks in medium with 10% FBS. Adherent cells in flasks were irradiated (2500 rads), removed from the flask by trypsinization and replated at a density sufficient to produce a confluent cell layer in the culture vessel. Feeder layers of bovine endothelial or mouse embryo (3T3) cells were less effective. We were unable to demonstrate survival-promoting activity in conditioned medium from the BRL cells, suggesting that the critical factor(s) provided by BRL cells may be unstable or that the feeder layer may function in other ways. For instance, feeder cells may remove a component from the medium that is detrimental to the worm cells or provide a necessary insoluble matrix not released into the medium.

Cells can be maintained for a least eight months on the feeder layers and for at least two months in the absence of feeder layers. This procedure leads to selection *in vitro* of clusters containing 20 to 200 juvenile worm cells. Plating on feeder layers also allows the extended maintenance of cell clusters derived from newly transformed schistosomula, although the fraction of cells surviving for more than two weeks in these cultures was less than that obtained with juvenile worms. Additional improvements in culture behavior were obtained by addition of serum albumin (probably acting as a source of lipid bound to the protein) and low concentrations of FBS. Recent modifications from those originally reported have also been made in levels of carbohydrates and amino acids, and thymidine is now used in the culture medium in place of thymine.

We identified and quantified the percentage of cells synthesizing DNA by following incorporation of the thymidine analogue 5-bromo-2'-deoxy-uridine (BrdU) into DNA with a specific monoclonal antibody to the nucleotide analogue. This procedure is commonly used

as an indicator of DNA synthesis in mammalian cells. For routine assay, medium was replaced by thymidine-deficient medium and 10 μ M BrdU added, cells were incubated for 4 hours and then fixed in cold methanol and air dried (Hobbs *et al.*, 1993). Fixed cultures were incubated with 2 N HCl (37 °C for 1 hour) to expose the nucleic acid sufficiently for reaction with antibody, neutralized, blocked with 3% BSA in PBS and incubated with fluorescein-conjugated anti-BrdU. The dye solution was replaced with PBS and fluorescence visualized with a fluorescein isothiocyanate filter. Nuclei of all cells in the culture also were simultaneously counterstained with propidium iodide to provide an indication of total cells, and cells undergoing DNA synthesis were quantified as a percentage of total cells in the culture. In experiments in which feeder layers were employed, nuclei of feeder layer cells were easily distinguished from those of schistosome cells because the latter are much smaller. Experiments evaluating percentage of cells incorporating BrdU and relative intensity of staining as a function of incubation time with BrdU have allowed us to differentiate between cells undergoing DNA replication vs those with background levels of DNA repair.

Quantitation of juvenile worm cells incorporating BrdU under assay conditions indicative of DNA replication showed 14.3% ($\pm$ 1.2%) in 1 day-old cultures, 8.0% ($\pm$ 3.6%) in 10 day-old cultures, 3% in 17 day-old cultures and 0.5% in 45 day-old cultures (expressed as percentage of total cells). To demonstrate that incorporation of the thymidine analogue BrdU reliably indicates DNA synthesis in these cells, we also carried out control experiments in which we found that incubation of fixed cultures with RNase-free DNase prior to exposure to anti-BrdU significantly reduced the fluorescent signal, while incubation with DNase-free RNase, did not affect the signal. In addition, the staining pattern we obtained with BrdU also argues for specificity of DNA stain: the signal was localized in the nucleus and was present only in a fraction of the cells, while one would expect RNA to be present throughout the cell and to be synthesized in every viable cell of the culture.

As cultures progress, they become predominantly cell aggregates. Observations over the first few days of culture suggest that some clumping of single cells is contributing to the appearance of the aggregates. However, by 10 days in culture BrdU incorporation was seen primarily in cell aggregates (Hobbs *et al.*, 1994), suggesting that cell proliferation is also contributing to the phenomenon. The percentage of BrdU-positive

cells diminished with time, but some aggregates in three to four week-old cultures contained up to 40% BrdU-positive cells, suggesting that cell proliferation continues in isolated areas of the culture. This nonrandom distribution, as well as precautions in assay and evaluation, suggests that incorporation is the result of DNA replication, rather than DNA repair, which might be expected to be more equally distributed throughout the cells of the culture.

Flow cytometry of freshly disaggregated cells stained with propidium iodide identified populations of cells in G1, S and G2 phases of the cell cycle in the starting material for the cultures, indicating that some cells were synthesizing DNA or committed to do so at the time of culture (Hobbs *et al.*, 1993). Similar populations were detected by flow cytometry of cultures after 17 days *in vitro*, also indicating that DNA replication was occurring in the cultures, although the S phase and G2 phase populations were reduced compared to the starting material.

Metabolic labeling of primary cultures with radioactive methionine followed by fractionation of cellular material by electrophoresis and autoradiography indicated that the pattern of protein synthesis in these early cell cultures was similar to that of intact juvenile worms (Hobbs *et al.*, 1993). Cell cultures from juvenile worms and control cultures of undisturbed juvenile worms were maintained for one week *in vitro*, at which time 35 S-methionine was added. After 24 hours cultures were harvested, washed and cells lysed in PBS with 0.1% Triton X-100. Equal amounts of radioactivity were subjected to SDS-PAGE (12% gel) and the dried gel was exposed to autoradiography.

As a further indication that the cultures faithfully represent the physiology of the organisms from which they were derived, we observed that the cultures exhibited a contractile response to serotonin added to the medium. Serotonin is known to function as a messenger molecule in *S. mansoni* via activation of a cellular signaling mechanism involving adenylate cyclase coupled through a G protein (Iltzsch *et al.*, 1992). Cells and cell clusters were cultured on a feeder layer for 4 weeks. To initiate the experiment, medium was changed and serotonin at various concentrations was added. In each of 4 wells, 5 randomly chosen, small adherent clusters (5–20 cells each) were observed for 30 sec each and the number of contractions of each cluster recorded. Responses were observed within 10 minutes after addition of serotonin and continued for 24 hours after addition. Maximum response was observed about 20 minutes after addition of serotonin to 400 ng/ml. Cell

clusters remained capable of contraction for as long as six months when cultured on the BRL feeder layer. Chronic addition of serotonin to the culture medium led to increased routine levels of contractility, but did not improve proliferation or viability of the cultures.

Culture of cells from the molluscan stage of the life cycle

We have also exploited the asexual developmental potential of the primary sporocyst stage of *S. mansoni* which derives from the miracidium after penetration into the intermediate snail host *Biomphalaria glabrata*. Sporocysts were chosen because they represent a relatively undifferentiated, proliferative phase that may be more like embryonic cells committed to unlimited cell division. Hamsters are used as the host for procurement of parasite eggs from which sporocysts are obtained. Eggs from the livers are hatched to produce miracidia which are then transformed *in vitro* to primary sporocysts, followed by mechanical and enzymatic disaggregation in a procedure similar to that used for juvenile worms. We also developed procedures for initiation of cultures from secondary sporocysts isolated from infected snails. Secondary sporocysts were derived by infection of snails with miracidia, dissection of sporocyst material from the hepatopancreas and multiple washings in antibiotic-containing medium, followed by mechanical and enzymatic disaggregation.

Chronic exposure to snail plasma *in vitro* was deleterious to schistosome cells, though this usually was eliminated if the plasma was mixed with dithiothreitol to 0.1 mM (Hansen, 1976). However, when miracidia or primary sporocysts were exposed to low concentrations of snail plasma for short time periods prior to disaggregation, the serum apparently provided a developmental signal to the schistosome and tegumental development was triggered, providing primary tissue fragments that stuck more readily and more avidly to the culture surface. On tegumental 'patches' thus obtained, cells may be cultured for three months or longer, and flame cells and serotonin-responsive muscular contractions are observed similar to those seen with cell clusters from juvenile worms.

Cultures were maintained in a mixture of equal volumes of the medium developed for the culture of juvenile worms (Hobbs *et al.*, 1993) and medium F (Stibbs *et al.*, 1979) which is N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid]-buffered and contains Basal Medium Eagle's vitamins, amino and organic acids, sugars and salts, glutamine, penicillin

and streptomycin (Bayne *et al.*, 1994). Also beneficial in this medium is lipid-enriched albumin and SF9 (insect cell)-conditioned medium. At the edges of fragments in the best cultures, bright turgid cells, both individual and clustered, were visible.

Sporocyst-derived cells and tissues were maintained best when FBS was present and the percentage of cells synthesizing DNA was quantitatively associated with the percentage of FBS in the culture medium. Best results were obtained with cultures maintained in media with 10% or more FBS (Bayne *et al.*, 1994). Synthesis of DNA in the cell cultures was assessed by incubation with BrdU followed by identification of cells incorporating BrdU using an enzyme-linked assay employing monoclonal anti-BrdU antibody. DNA synthesis was detected primarily in clusters of cells and DNA synthesis occurred to a greater extent when cultures were initiated at high cell densities. Indications are that the cells in the cultures incorporating BrdU into DNA are germinal cells because in similar experiments with intact sporocysts the cells that are positive in this assay lie within the mesodermal parenchyma, mostly in the posterior half of the organism, are small, and number 20 or less. These characteristics are consistent with those reported for germinal cells as described by Pan (1980). Metabolic labeling with radioactive methionine and electrophoretic analysis as described for juvenile worm extracts demonstrated a protein synthesis profile in cell culture similar to that of intact sporocysts, and the amount of phosphotyrosine-containing protein in the sporocyst cells or intact, newly transformed sporocysts was low, similar to that of juvenile worm cells.

Recently other laboratories have obtained evidence for the existence of schistosome and host snail neuropeptides that engage in host-parasite, bidirectional communication, and for molluscan-derived growth promoting peptides with mammalian homologues (Smit *et al.*, 1988; Duvaux-Miret, 1992; Solis-Soto and de Jong-Brink, 1994). Coculture with ganglia taken from uninfected and infected host snails (*B. glabrata*), and with cells of the Bge line (ATCC CRL 1494), yielded culture that retained healthy appearances for longer than axenic controls (Bayne *et al.*, 1994). The Bge line (Hansen, 1976), derived from embryonic cells of *B. glabrata*, is maintained in Hansen's original medium with selected FBS at 10% and added phenol red. *B. glabrata* circum-esophageal nerve rings containing all major snail ganglia were dissected from alcohol-swabbed adult snails. Surgery was performed under medium F containing penicillin,

streptomycin and amphotericin B. The body wall was opened over the head, the esophagus cut and pulled forward through the nerve ring, and the ring removed after cutting the nerves connecting it with other tissues. Nerve rings were washed 3 times in the antibiotic-rich medium F before being placed in wells in schistosome medium.

Beneficial effects of snail-derived cells were not as obvious when the cocultured material was held in well inserts in which a molecularly-permeable membrane separated the schistosome cells from the snail cells; actual contact may be necessary. Addition of media conditioned by Bge cells did not prove to be profitable. Bge-conditioned medium was obtained by replacing the Bge medium with a 1:1 mixture of schistosome medium and Hansen's 'synxenic medium' (Hansen, 1976) the day before use. The Bge cells survived well for over a week in this medium. Co-culture with snail nerve cells in particular leads to prolonged maintenance of healthy cultures, and our results with co-cultures suggest that host-derived neuronal products may serve as trophic agents for schistosome development.

Discussion

Thus far we have derived cultures of cells from *S. mansoni* exhibiting long term viability and limited proliferation. Our approach has been to develop culture methodology for cells from both juvenile worms (mammalian host stage of the life cycle) (Hobbs *et al.*, 1993) and sporocysts (molluscan host stage of the life cycle) (Bayne *et al.*, 1994). We chose these stages because they represent periods in the life cycle of the parasite during which active cell proliferation is occurring, thus maximizing our chances of obtaining proliferating cells in the primary cultures. In addition, juvenile worms were chosen because their physiological milieu is that of mammalian cells, for which culture methodology is well developed (Barnes, 1987).

Continuously proliferating cell lines from this organism will provide new avenues toward studies of nutrition, physiology, endocrinology, immunology, pharmacology and toxicology of parasitic disease. Such lines will provide an accessible source of parasite material for laboratories that do not have facilities and resources to maintain the complex life cycle of the organism, and our approaches may be applicable to *in vitro* culture of cells from other helminths and other lower invertebrates. These applications may pro-

vide additional experimental tools in nonmammalian models of developmental biology, neurobiology and other basic biological disciplines that are relevant to biomedical research in general. In addition, advances in understanding the molecular biology of *S. mansoni* and other helminths, including identification of probable promoter sequences, growth-related genes and processing pathways (Rajkovic *et al.*, 1990; Shoemaker *et al.*, 1992; dos Reis *et al.*, 1993) suggest the potential to directly alter the genomic makeup of the cells, opening additional experimental avenues toward mechanistic explanations of physiological phenomena.

Furthermore, the long term potential exists through our proposed experiments to select for sporocyst germinal cells that have been genetically manipulated *in vitro* (i.e., by addition or disruption of genes) and introduce them into attenuated (eg., by radiation) mother or daughter sporocysts so that cercaria derived from the manipulation contain the genetic alteration introduced *in vitro*. This would be a powerful technique for identifying genes critical to survival, growth and pathogenicity of the organisms. Similarly, a cell culture system is needed to study trans-splicing processes (Rajkovic *et al.*, 1990), and cultures from *S. mansoni* likely would satisfy this need.

Potential also exists for the use of cell cultures of *S. mansoni* for vaccine purposes (Weller and Weeldon, 1982). While considerable effort has been directed to the use of recombinant antigens in recent years, biologically-derived material and attenuated organisms (eg., irradiated cercaria) are also recognized as highly protective (Mountford *et al.*, 1995). Immunogenicity of irradiated cercariae may be due partially to the site of presentation of antigen, but also is probably related to the more biologically-relevant state of the immunogen. It has been suggested that exposure to specific antigens characteristic of early stages in the development of the worm in the mammalian host may be particularly important for protective immunity. In this regard our cell cultures derived from juvenile worms may provide the appropriate antigens (Harrop and Wilson, 1993).

Optimum protective immunization may require exposure to cell-derived glycoprotein, lipoprotein or glycolipid complexes that can not be mimicked by single recombinant products but might be well presented by cultured cells. It is known, for instance, that novel, immunogenic surface glycosphingolipids and novel antigenic glycoprotein carbohydrates exist in *S. mansoni* (Makaaru *et al.*, 1992; Srivatsan *et al.*, 1994; Bergwerff *et al.*, 1994). We feel that the potential for large scale, economically feasible production of pro-

tective immunogen from cell cultures of *S. mansoni* merits further investigation. We are particularly interested in pursuing the use of the cultures described here as potential vaccines, as experimental models for drug screening and genetics of drug resistance, and in the study of hormonal signalling mechanisms in these parasites.

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