QUANTITATIVE ASSESSMENT OF MARINE SPONGE CELLS IN VITRO: DEVELOPMENT OF IMPROVED GROWTH MEDIUM

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SUMMARY

As sources of natural products with potential human therapeutic value, marine sponges are important subjects for cell culture studies. A critical component of any cell culture system is its growth medium. Proceeding from the hypotheses that the thawed, cryopreserved, primary cells would display detectable differential responses and that those responses could be comparatively quantified, this study has established that multiwell screening assays are useful tools for improving medium formulations in cell cultures of the marine sponge, *Teichaxinella morchella.* Fluorescent probe signals were correlated with known cell densities and viabilities in a 96-well format. Analysis of variance and post-test methods were applied to judge the significance of signal differences seen in a variety of medium formulations. Results from a series of experiments suggested that reducing glutamine and selenium concentrations in the standard medium would result in greater DNA, protein, and esterase activity signals. This was confirmed by the direct comparison of the standard and improved medium formulations. Significantly higher protein content and esterase activity were associated with the improved medium. DNA content was also higher, though not significantly. The result is a new medium formulation that may be more able to support cell growth and division, providing an improved cell culture system for marine sponge cell studies. The assays can be used in additional studies to further improve the in vitro conditions for marine sponge cell culture.

Key words: sponge; cell culture; Hoechst; fluorescein diacetate; sulforhodamine B; Porifera; invertebrate.

INTRODUCTION

Despite intense interest in the in vitro behavior of marine sponge cells, there is no reported continuously replicating marine sponge cell line. Such a cell line could be an important tool for both basic research in marine invertebrate cell biology and for developing methods for the production of sponge-derived natural products with human therapeutic value. Only a modicum of success has been achieved by investigators attempting to culture sponge cells and the cells of marine invertebrates in general. Contamination is an ongoing concern, as evidenced by the ultimate identification of some cultures as protozoans (Klautau et al., 1994). While primary cell cultures from marine sponges have been initiated (Klautau et al., 1994; Pomponi and Willoughby, 1994; Rinkevich et al., 1998), little research has been done on nutritional requirements for growth of marine sponge cells in vitro. Though much has been learned about signal transduction pathways in relation to cell aggregation (Muller et al., 1990) and, to some extent, mitogenesis (Gramzow et al., 1989), few studies directly address the question of optimal composition of the liquid media in which the sponge cells are maintained. The question of cell attachment was addressed by Gaino et al. (1993) and Leys (1997), who documented changes in cell morphology according to the substrate. Gramzow et al. (1989) monitored cell proliferation, and Schroder et al. (1989) documented the *ras* gene expression in response to homologous lectin. However, only Sorokin et al. (1993), Ilan et al. (1996), and Pomponi et al. (1994, 1997a, 1997b) have manipulated the nutritional environment and recorded the resultant sponge cell number increases and changes in sponge cell viability. The lack of research in this area represents a notable omission in the field of marine sponge cell culture because even an immortalized cell line will require an appropriate cell culture medium to sustain growth.

The research described here has been aimed at developing nonradioactive, automated, multiwell plate assays for the assessment of sponge cell responses to nutritional parameters. Proceeding from the hypotheses that the cells would display differential responses and that the responses could be measured and comparatively quantified, assays were developed to accomplish three objectives: to compare DNA contents of cultures, to compare cell number or size changes via protein content, and to detect differences in metabolic activity. The assays provide methods for rapidly evaluating the effects of variables on multiple samples of dissociated sponge cells. The result is a reduction in the time and effort required to obtain statistically significant data from large sample numbers and multiple variables, facilitating complex studies such as growth medium optimization. This utility was applied to analyze an existing growth medium known to support the growth of sponge cells from multiple species. The medium formulation is a combination of several components, any one of which may or may not be advantageous or

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inhibitory. In order to optimize the medium formulation, individual components must be added, removed, or applied in alternative concentrations before testing the cell response (Ham and McKeehan, 1979). Any of these strategies requires multiple-sample comparisons, demanding rapid, objective procedures for collecting and analyzing large amounts of data. This study developed appropriate procedures for these tasks. Accordingly, data presented here represent a step toward improving in vitro conditions for cultivation of marine sponge cells.

Materials and Methods

Sponge cells and standard growth medium. Cells were obtained from cryopreserved stocks. All were derived from *Teichaxinella morchella* (Porifera, Demospongiae, Axinellida, Axinellidae) specimens collected in the waters of Florida, the Bahamas, and the Caribbean Sea, and were cryopreserved according to Pomponi et al. (1997a). Multiple cryopreserved cell lots, derived from different sponge specimens, were used in order to provide the cell numbers needed for the assays, though no single experiment required more than one lot of cells. *Teichaxinella morchella* is a good model for studies relating to natural products because it produces the antitumor compound stevensine (Albitzi and Faulkner, 1985). It is relatively common and easy to collect, amenable to culture, and produces the bioactive compound in vitro (Pomponi et al., 1997a). Vials of cryopreserved cells were thawed quickly in a 50° C water bath and held on ice. The cell suspension was rinsed and resuspended in Medium 199–based sponge cell culture medium with rifampicin and amphotericin B (Pomponi et al., 1997b), hereafter referred to as the standard growth medium (SGM). A portion of the final suspension was diluted in calcium- and magnesium-free artificial seawater (CMF) for counting and viability assessment with trypan blue. Except when otherwise indicated, all cell preparations exhibited a postthaw trypan blue viability rate of 70–75%.

DNA assay. Hoechst 33342 (H33342) is a stain that enters live cells and, with ultraviolet (UV) excitation, fluoresces in the blue range on binding to the minor groove of DNA. It has been used in a multiwell assay format by McCaffrey et al. (1988). For this study, the dye (Molecular Probes, Eugene, OR; H-3570) was diluted to 40 ng/ml in CMF. Multiwell plate cultures were rinsed twice with 250 μl CMF per well by centrifuging and decanting the supernatant. After the final rinse, diluted H33342 (250 μl per well) was added. Plates were incubated in the dark at room temperature for 1 h. Signals were read with a Dynex Fluorolite 1000 fluorescence plate reader, using 350-nm (30 bandpass) excitation and 450-nm (65 bandpass) emission. The plate reader voltage was kept at 8.5 V, with reference channel number 3 set at 1375 fluorescence units (arbitrary units). Linear correlation analysis was performed on results for well cultures with a range of cell densities from 2.5×10^5 to 1.5×10^6 cells/ml. Eight replicate cultures, in SGM, were prepared for each density. The correlation between cell density and fluorescence was analyzed in order to yield a coefficient of determination.

Protein assay. The protein assay, a modified version of the one reported by Skehan et al. (1990), is based on electrostatic binding of sulforhodamine B (SRB) to basic amino acid residues. After two plate rinses, as described earlier, 200 μl CMF was added to each well. The plate was then centrifuged to pellet any suspended cells. Fixation was performed by layering 75 μl 80% cold trichloroacetic acid in cold CMF onto the contents of each well. The plate was incubated at room temperature, undisturbed for 30 min, and then refrigerated for 90 min. After decanting the acid solution, the plate was rinsed five times with deionized water gently poured from a beaker, inverted, and blotted on a paper towel. Air-drying was accomplished by inverting the plate at a slight angle overnight. SRB (Sigma Chemical Co., St. Louis, MO; S9012) was prepared as a 0.8% solution in 1% acetic acid. The dry plate received 50 μl SRB solution per well and was incubated at room temperature for an hr. After decanting the stain solution, the plate was rinsed five times with 1% acetic acid poured from a beaker and dried as described earlier. The stain was solubilized with 100 μl per well 10 m*M* unbuffered Trizma base (Sigma). After 10 min of gentle shaking, the plate was read using 455 nm (70 bandpass) excitation and 580 nm (10 bandpass) emission. The standard instrument setting was 8 V with reference channel number 1 set at 396 fluorescence units (arbitrary units). However, these settings had to be modified slightly in some cases to accommodate unexpectedly high signals. Linear correlation analysis was performed on results for well cultures with a range of cell densities from 0.5×10^5 to 1.25×10^6 cells/ml. Eight replicate cultures, in SGM, were prepared for each density. The coefficient of determination was calculated.

Esterase activity assay. Nonspecific esterase activity was measured with fluorescein diacetate (FDA), which has been previously applied to marine sponge cells (Yentsch and Pomponi, 1994). This nonfluorescent compound is cleaved by esterases in metabolically active cells into fluorescein (Dorsey et al., 1989). The stain stock was prepared at 5 mg/ml in dimethyl sulfoxide. A fresh working solution was prepared by diluting 1 part stock in 1000 parts fetal bovine serum-free SGM. After rinsing the plate twice as described earlier, 250 μl of working solution was added to each well. The plate was incubated in the dark at room temperature for 1 h and read using 455 nm (70 bandpass) excitation and 535 nm (35 bandpass) emission. The voltage was set at 3 V with reference channel 4 at 2011 fluorescence units (arbitrary units). A linear correlation analysis was performed on the results for sets of five replicate well cultures with a range of viabilities in SGM. The coefficient of determination was calculated.

Medium optimization. The SGM formulation contains additives, including fetal bovine serum (FBS), glutamine, and selenium, as supplements to the basal nutrient mixture. These supplements were tested in the three assays to determine if they produced any effects. Wells of the Falcon Primaria 96-well plates contained 250 μl of cell suspension with 5×10^5 viable cells/ml. Phytohemagglutinin (Gibco, Gaithersburg, MD; 10576-015) was added, 1.5% by volume, to stimulate cell division (Pomponi and Willoughby, 1994). Plates were assayed after 36 h of incubation at 20° C based on previous observations of cell doublings after approximately equal times (Pomponi et al., 1997b).

Data management. Five replicate test wells were prepared for each treatment along with an equal number of cell-free control wells containing the same treatment medium. The mean cell-free control well reading was subtracted from each test well reading in order to arrive at a value corrected for any differences due to the medium. Statistical tests were performed as recommended by Zar (1974). Calculations were performed by GraphPad InStat (1998). The means of the adjusted values were compared by one-way analysis of variance, followed by the Tukey multiple-comparisons test. The Bartlett method was used to test for significant variations among standard deviations. The data were also subjected to a normality test using the method of Kolmogorov and Smirnov. All data displayed normal distributions and equality of variances. For comparison of the standard and improved media, a one-tailed *t*-test was used. The 0.05 maximum *P* value convention was used as a guide for statistical significance.

Viability comparison. Because the proportion of dead cells can vary among samples, and initial viability could influence assay results, an FBS experiment, like the one described later, was run with two different cell samples with post-thaw viability rates of 32 and 62% (trypan blue exclusion). All samples were contained in the same well plate and measured simultaneously with identical reagents. A DNA assay was run to determine if a low initial viability would significantly affect conclusions drawn from signals measured after 36 h.

Fetal bovine serum study. For some cell lines, FBS is a source of beneficial substances such as growth factors (Freshney, 1994). Because FBS is an undefined, complex, and expensive additive, its utility was investigated. SGM was prepared without FBS. Cells were suspended in this medium at an elevated density so that the addition of SGM–FBS solutions would result in the desired initial cell density. FBS was tested at 0, 5, 10, 15, and 20% by volume.

Glutamine study. Though glutamine is included in many mammalian cell culture media (Freshney, 1994), its importance in sponge cell cultures is not established. In order to determine associated effects, SGM was prepared without glutamine. A glutamine solution was used to adjust levels to the standard 50 μg/ml concentration for SGM (1×) and to concentrations of 0 (0×), 5 (0.1×), 25 (0.5×), and 500 (10×) μg/ml.

Selenium study. Though selenium might offer antioxidant benefits, it could also be toxic at inappropriate doses (Freshney, 1994). In order to investigate its effects, SGM was prepared without selenium. A solution of sodium selenite was used as described for glutamine, with 18 ng/ml as the standard SGM (1×) concentration.

Comparison of standard and improved media. Data from the above experiments were used to design a medium, incorporating features associated with improved results, i.e., higher DNA and protein contents, and greater esterase activity. The improved medium had the standard 5% FBS concentration, one-half (0.5 ×) the standard glutamine concentration (25 μg/ml), and one-tenth

(0.1 ×) the standard selenium concentration (1.8 ng/ml). All cultures were incubated for 36 h before running the assays.

Results

Correlation analyses. Studies performed on known gradients of cell density and cell viability indicated that assays would be useful for detecting differences among cell populations. For DNA, protein, and esterase activity assays, the coefficients of determination were, respectively, 0.9139, 0.7766, and 0.9683. In all cases, $P < 0.0001$.

Control data considerations. All cell-free control data sets exhibited normal distributions according to the Kolmogorov and Smirnov method and no significant differences among standard deviations according to the Bartlett test. Differences among treatment controls were significant in three cases. Increasing the FBS concentration caused signal elevation in both the DNA and protein assays. This was expected because of the inherent UV fluorescence and protein content of the serum. The esterase activity assay was significantly affected by the highest concentration of selenium, which produced higher control signals than all other concentrations. Either the selenium itself contributed a detectable signal, or the noncellular spontaneous reaction was promoted. Because all final data were adjusted by subtraction of control values, the contribution of these differences was minimized. Furthermore, the conclusions drawn from the actual tests do not appear to be based upon such background differences, since increasing FBS and the highest concentration of selenium were not associated with improved results.

DNA and protein assays. The results of the DNA and protein assays were somewhat different than expected, but the combination of the two assays still served the purpose of elucidating detectable differences in one assay when the cells failed to respond in the other. The significant relative protein increase in some cultures that do not demonstrate significant DNA increases was unexpected. On the basis of previous experiences with these cells, which often appear to remain unchanged in size for long periods in culture, we predicted that nondividing cells would maintain an essentially static protein content. However, the data indicate that cells were indeed able to display significantly elevated relative protein content without significantly elevated relative DNA content, indicating possible increases in cell size without cell division, as would be generally anticipated in cell culture. This may represent a previously undetected measurable type of sponge cell growth that could become a significant aid in assessing responses of noncycling sponge cells. Furthermore, such relative protein increases might represent differential expression of specific proteins.

Esterase activity assay. The esterase activity data yielded the least significant results. This was also unexpected, since this general metabolic measurement was predicted to be most useful if cells failed to demonstrate increases in relative protein and DNA contents. Esterase activity was anticipated to fluctuate more than the other two parameters. Because this assay is most obviously a measure of viability and, perhaps, an even more sensitive indicator that can detect changes not associated with actual cell death or lysis, the results may indicate that the viability and general metabolic condition of the cells are unaffected by most of the applied treatments. In each experiment, there was a trend of decreasing esterase activity over time, though this is merely an observation and not statistically significant. It may be that some nonviable or marginally viable cells contribute to the overall esterase activity for a short time after inoculation, but cease to contribute after 36 h. These

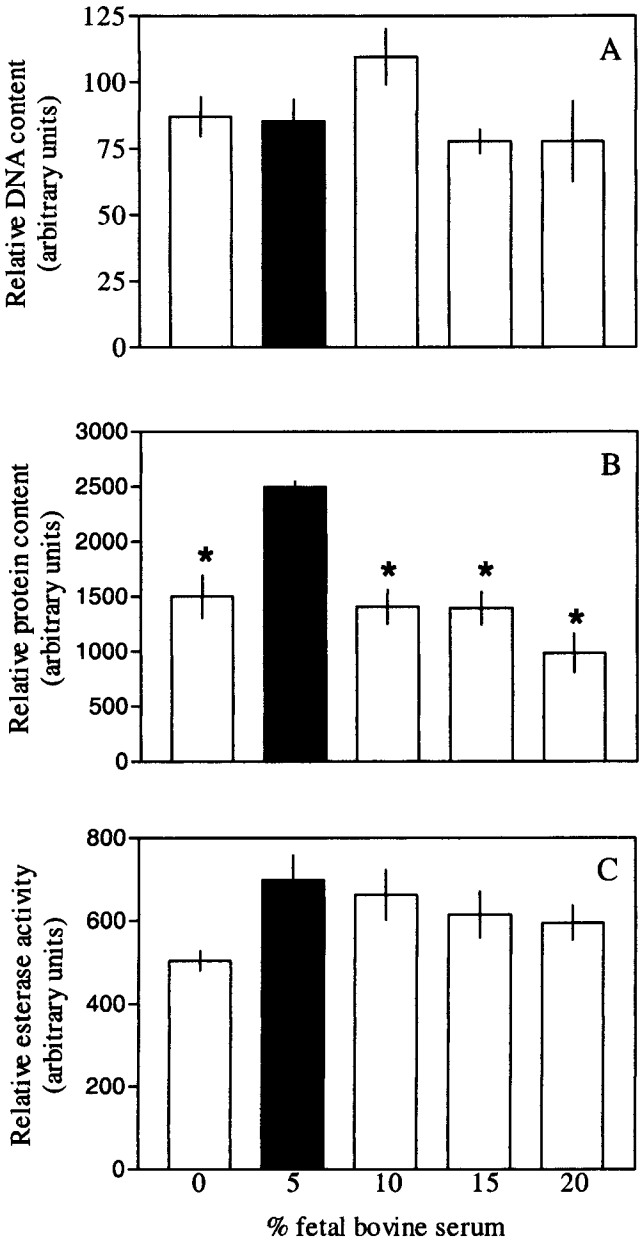

FIG. 1. Effect of fetal bovine serum after 36 h of incubation. (*A*) DNA content. (*B*) Protein content. (*C*) Esterase activity. Mean ± SEM, n = 5. *Solid bar* indicates standard treatment. Means marked with an *asterisk* differed significantly ($P < 0.05$) from the standard treatment mean.

dead or dying cells could contribute signals in the DNA and protein assays for a limited period of time. Beyond this observed tendency for the signal to decrease over time, the general trends in the FDA assays, though not significant, were similar to those seen in the other assays.

Medium optimization studies. Application of the three assays to cultures incubated in varied medium formulations yielded some significant results (Figs. 1–4). Although the total DNA content of cultures generally did not appear to increase over time, there were significant differences among the final net DNA contents for some treatments (Fig. 2*A*). Esterase activity tended to decrease with time,

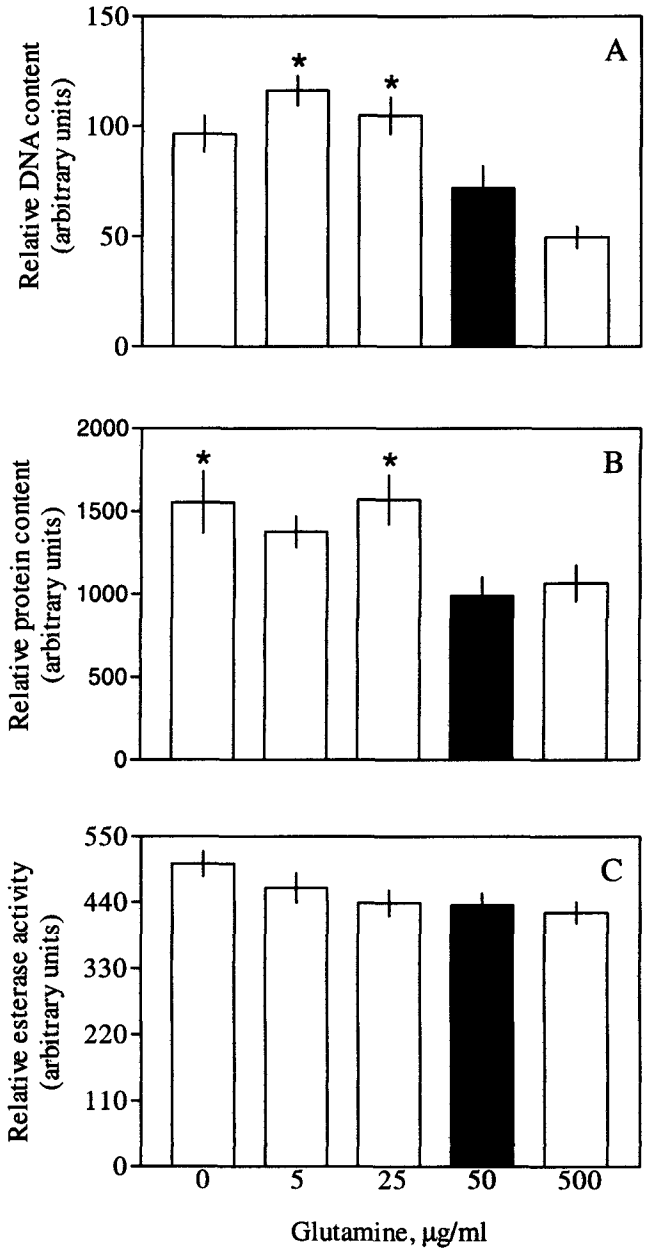

FIG. 2. Effect of glutamine after 36 h of incubation. (*A*) DNA content. (*B*) Protein content. (*C*) Esterase activity. Mean ± SEM, n = 5. *Solid bar* indicates standard treatment. Means marked with an *asterisk* differed significantly ($P < 0.05$) from the standard treatment mean.

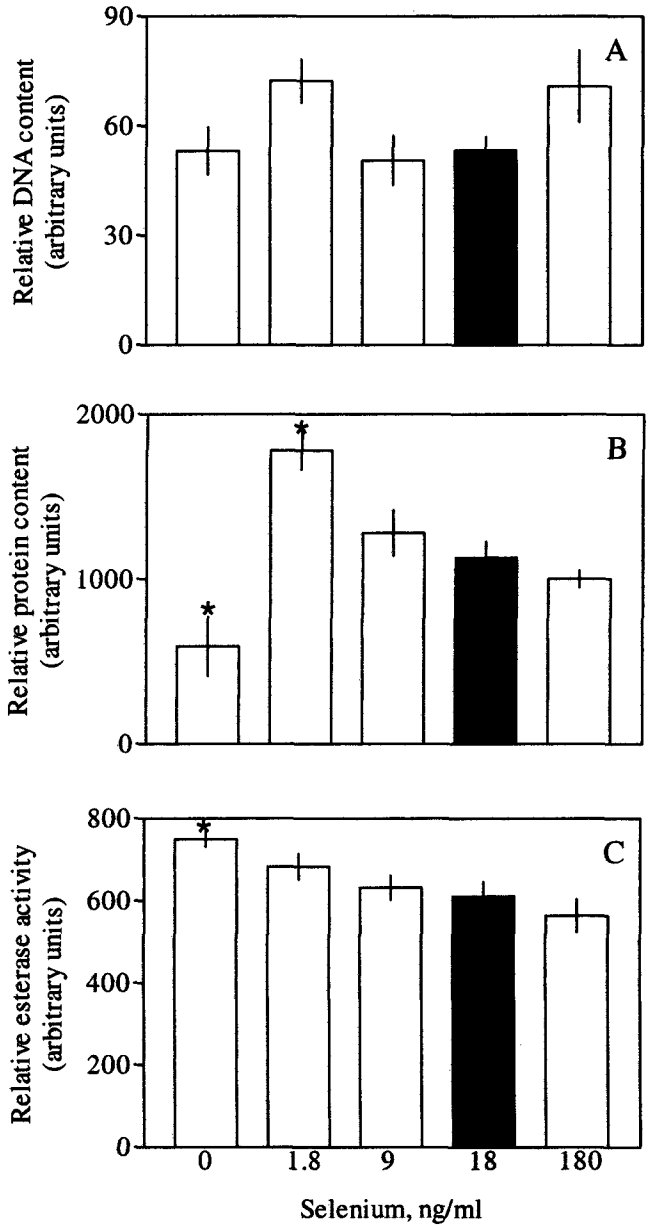

FIG. 3. Effect of selenium after 36 h of incubation. (*A*) DNA content. (*B*) Protein content. (*C*) Esterase activity. Mean ± SEM, n = 5. *Solid bar* indicates standard treatment. Means marked with an *asterisk* differed significantly ($P < 0.05$) from the standard treatment.

whereas protein content tended to increase. These temporal trends are noted merely as observations and should not be considered statistically meaningful.

FBS. There were no significant differences in the effects of FBS on mean total DNA (Fig. 1*A*). There were, however, differences in protein content, with the FBS concentration of 5% producing the highest protein signal (Fig. 1*B*). After 36 h of incubation, all other treatments resulted in significantly lower protein signals (Fig. 1*B*). While screening methods such as these are not designed to determine the reasons for the differences observed, it might be hypothesized that FBS contains some beneficial components, but one or more components may become inhibitory at higher concentrations. Esterase activity did not appear to increase over time, and there were no significant differences in esterase activity among treatments (Fig. 1*C*). These data suggest that none of the alternative FBS concentrations offer any advantage over 5%. On the basis of the results of the protein assay, the 5% concentration was determined to be optimal for purposes of this study.

Glutamine. Statistically significant high DNA and protein contents were associated with reduced glutamine concentrations. Relative glutamine concentrations of 0.1× (5 µg/ml) and 0.5× (25 µg/ml) resulted in significantly higher DNA contents than the standard treatment (Fig. 2*A*). Both omission of glutamine and glutamine at

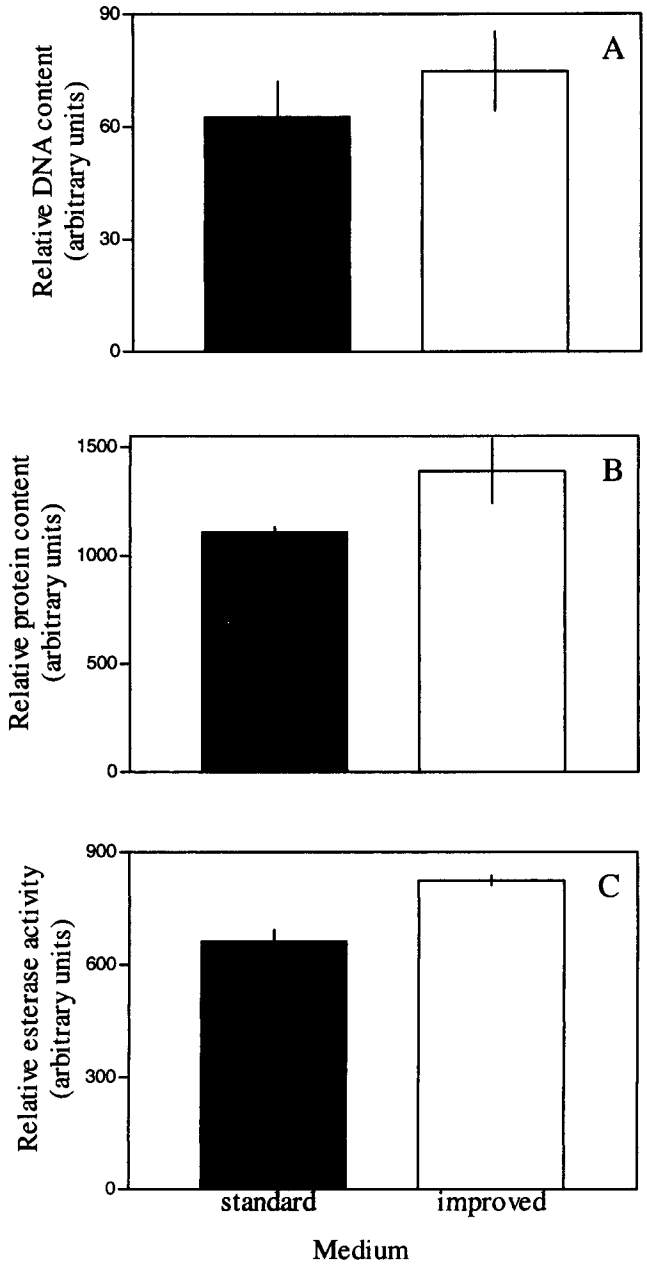

FIG. 4. Effect of improved medium after 36 h of incubation. (*A*) DNA content ($P = 0.2072$). (*B*) protein content ($P = 0.0499$). (*C*) Esterase activity ($P = 0.0006$). *Solid bar* indicates standard treatment. Mean ± SEM, n = 5.

25 μg/ml resulted in protein contents higher than the standard treatment (Fig. 2*B*). The concentration of glutamine was not related to any significant difference in the esterase activity (Fig. 2*C*). On the basis of favorable results in two of the three assays, the conclusion, for purposes of this study, was that the glutamine concentration of 25 μg/ml was optimal.

Selenium. The selenium study produced total DNA contents that were not significantly different from the standard treatment signal (Fig. 3*A*). However, notably significant differences in protein content were seen, with a 0.1× relative selenium concentration (1.8 ng/ml) resulting in a protein content higher than the standard treatment and omission of selenium resulting in a protein content lower than the standard treatment (Fig. 3*B*). Increased esterase activity, relative to the standard treatment, was seen with omitted selenium (Fig. 3*C*). On the basis of these results, 1.8 ng/ml selenium was designated as optimal.

Comparison of standard and improved media. For all three assays, higher signals were associated with the improved medium containing lower concentrations of glutamine and selenium. The differences were statistically significant for protein and esterase activity, but not for the DNA assay, which yielded a P value of 0.2072. The data are summarized in Fig. 4*A–C*.

DISCUSSION

Data presented here support the hypotheses that marine sponge cells can display detectable, measurable responses to the in vitro environment that can be comparatively quantified in a multiwell format and used to guide culture medium optimization studies. Objectives for development of assays were met by establishing correlations between each of three stain signal intensities and their respective targets in samples of known cell density or viability. The assays were used to measure differential responses to nutritional parameters by detecting significant differences among cells incubated in various media.

The procedures developed in this study exhibit many of the advantageous features offered by screening methods used in more conventional mammalian in vitro bioassays. Furthermore, they offer new methods for examining broad ranging effects of diverse treatments on cells not previously studied in this manner. The large-scale systematic analysis of in vitro effects of medium components is a notably novel approach in the field of marine sponge cell culture. Because little is known about the in vitro cellular physiology of sponges, this research represents a significant step toward understanding basic sponge cell biology. The ability to test the effects of many variables by efficient and objective means allows the investigator to conduct expansive searches for what may be elusive clues.

Though it is not a routine practice in high throughput screening programs, it is prudent to interpret the results of each experiment independently. In this study, in particular, it is probably not valid to draw strong conclusions about differences between time points. For example, if a signal for a given treatment is higher after 36 h than it was soon after inoculation, it is not valid to automatically assume there has been an increase in the assay target molecules. Several sources of experimental error could cause such an effect. Though such pitfalls are common features of high throughput screening procedures, they are usually disregarded with the understanding that they exist, but are not likely to frequently contribute a significant influence in a system (i.e., mammalian cell bioassays) that produces robust differentials. In studies such as the present one, in which the cells do not respond as vigorously as the mammalian cell lines typically used in high-throughput screening procedures, the differences being measured are often very small. It is therefore important to consider such sources of variation. Furthermore, for this particular study, the plate reader settings for the protein assay had to be modified, in some instances, to keep unexpectedly high signals within the functional range of the instrument, precluding plate-to-plate analyses. Thus, formal comparisons have been limited to those wells contained within a single plate. The

data within a single figure in this study represent results from a single plate and can be legitimately compared.

In addition to the above cautions, the viability of the cells at the time of inoculation must be considered when interpreting these data. With viability rates of 70–75%, each well contained approximately 25% dead cells at the time of inoculation. Since many of the dead cells could lyse and essentially be eliminated from the population, differences in endpoint signals may be due to the loss of cellular material as well as the addition of new cells and materials. This means that there is likely a bidirectional influence on the net dynamics of each sample. Each of the assays measures only the endpoint, so the contributions of losses and gains cannot be individually determined.

There are other important sources of variation in the material used to initiate cultures. The sponge cells are cryopreserved in lots; each lot comprised vials of essentially identical cell suspensions taken from a single individual sponge. Since there were not enough cells in a lot to perform this entire series of experiments, data from many different lots are represented here. The possibilities for variation are therefore great, as the individual sponges were collected from different sites, depths, and temperatures. Some specimens were collected by submersible, others by hand using scuba, so the handling of specimens during and after collection varied. The individual specimens had varied morphologies, cell type distributions, reproductive states, sizes, ages, and associated microbial populations. Because no sponge cell line is available, this major source of variation between experiments is as yet unavoidable. Though error sources such as these complicate the analysis of multiwell screening data, they are traded for the strengths mentioned earlier, namely their ability to elucidate trends that can be selected for further study. Consequently, screening methods developed in this study are conservatively interpreted, considering only results within single plates, and have produced meaningful results when applied to marine sponge cell culture growth medium optimization.

These experiments were done with *T. morchella* cells. Results with other sponge species may vary. In addition, results can vary among specimens of the same species. For these reasons, generalizations based on a limited number of experiments should be avoided. It is important to bear in mind that the methods described here were developed for screening purposes. They are not intended to give definitive, comprehensive answers to questions about sponge cells in vitro but rather to survey large number of treatments in order to uncover potentially differential responses that can be studied later in greater detail. Future research may demonstrate responses common to all sponge specimens of all species, but for now, such generalizations should be avoided.

This study was conducted with primary cell cultures, not a cell line. The cultures are not axenic. Though steps were taken to reduce contaminants during the cell preparations before this study, the cultures undoubtedly contain some contaminants. Whole sponges in nature host populations of bacteria, fungi, algae, and protozoans that can comprise significant portions of the total tissue volume. Gradient centrifugation is used to reduce the number of contaminating cells. However, putative microbial contaminants are probably not entirely eliminated. Antibiotics and antimycotics are used to prevent the growth of such organisms (Pomponi et al., 1994). Our extensive previous experience with *T. morchella* suggests that associated microorganisms are not significantly affecting the assays described here. When contamination does appear to be a problem, it typically exists as a single fungal focus in a high volume long-term culture, suggesting that fungi are either well controlled by the antimycotic or sponge metabolites or are present in very low numbers. Bacterial contamination appears to be well controlled by rifampicin, as even very old cultures do not display any sizeable bacterial populations. Though it is possible for endosymbiotic bacteria to escape detection, microscopic examination suggests that the sponge cells tend to persist in culture with no notable concurrent increases in other cell types. Certain protozoans and thraustochytrids can be mistaken for sponge archaeocytes (Rinkevich, 1999), but suspicious cells are easy to detect with experience, and these contaminants are a relative rarity and not likely to overtake a culture in 36 h. However, the possibility of contamination is an ongoing concern, as it is in any cell culture system.

It is interesting to note that most researchers working with established cell lines do not take steps to confirm the identity of their cells. They tend to assume that the cells they started with are the same as the endpoint cells, even though contamination by similar cells is possible. Such contamination concerns have been continuously addressed during this study, although cell type verification was not the focus of this research. Molecular methods are being developed in our laboratory for detection of contaminated cultures. Flow cytometry is also used to contrast DNA histograms associated with characteristic relative DNA contents for different sponge species (unpublished data).

None of these methods was appropriate for use in this study. It is impractical to run any cell identification test on the 96 individual cultures in a multiwell plate. Each plate is scanned for any differences in well turbidity that might indicate contamination, and random rows or columns of wells are examined microscopically to detect any contamination patterns. Though these methods are not fail proof, three other factors combine to further reduce the probability of contamination affecting assay results. First, the cultures are incubated for only 36 h. This does not provide much time for a few cells to undergo cell division sufficient to contribute a large endpoint signal. Second, the individual culture volume is small, reducing the chance that a resistant contaminant will be present in any one culture well. Third, replicate cultures and statistical methods are used to assess the significance of any conclusions. Still, until there is an axenic sponge cell line available, the possibility of contamination will challenge all sponge cell culture research. Meanwhile, preventative measures such as antibiotics, along with careful experimental design, allow research to progress.

Progress in two key areas of knowledge will permit refinement of these assays as well as enhance their application. First, it is important to learn more about the condition of the cells prior to experimentation. The cells are subjected to a series of traumas during collection, transport, dissociation, gradient fractionation, and cryopreservation. They are then cultured in the presence of antimicrobial agents. These factors no doubt affect their performance in culture and may be linked to the occasional lack of increases in DNA over time, as seen in this study. Some or all of the cells may have been permanently or temporarily damaged, or signaled to initiate apoptosis. Cells have been observed to be fragile during the thawing procedure. The cell suspensions used for these experiments include both viable and nonviable cells (as measured by trypan blue exclusion) after thawing. Those that survive may be variably compromised, certainly not an ideal situation for experimentation. Undoubtedly, the preparatory procedure could be improved to limit

cell damage and improve lot-to-lot consistency. Currently, the assays are likely measuring the effects of treatments on variably traumatized cells with limited replication potential. Ideally, the assays will eventually measure the effects of treatments on undamaged cells with consistently high viability.

Second, it is imperative to understand more about the marine sponge cell cycle. Currently, timing of assays is based upon observations of cell doubling in cultures with mixed sponge cell types. Nothing is known about the schedule of DNA synthesis and cell division in any of the cell types. Such knowledge will permit more precise scheduling of assays. It will also introduce the possibility of controlling the cell cycle for experimental purposes. Elucidation of details of the cell cycle will contribute to the analysis of any apoptotic events that may be occurring during cell preparation or at any point in the culture process.

Thus, each of these areas of knowledge will provide information on the other. They illustrate the central problem in cell culture research lacking a cell line. In brief, the problem is that a cell line is required to properly do much of the research that would accelerate the development of a cell line. This dilemma is a significant one that challenges the creative development of techniques that will yield information in the absence of a cell line.

This study has established that multiwell screening assays are useful tools for improving medium formulations for cell cultures of the marine sponge, *T. morchella*. The result is a new medium formulation that is better able to support cell growth and division. Such improvements in medium composition may contribute to the ultimate goal of development of a cell line, in addition to raising and answering other questions about marine sponge cell biology.

Acknowledgments

We thank Drs. Julia Grimwade and Bill Baker (Florida Institute of Technology), and Dr. Clay Cook (HBOI) for their review of this work, and are grateful for the assistance of James Fredlock. Funding was provided by a Florida Sea Grant Marine Biotechnology award R/LR-MB-5. This is HBOI contribution 1317.

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