ABSTRACT

Human breast carcinomas have been one of the most difficult tumor types to culture in agar-based clonogenic assays. This fact has limited their clinical applicability. We have used statistically motivated experimental designs to systematically improve the clonal culture of enzymatically monodispersed primary human carcinoma cells in an anchorage-independent agar system. Based upon an initial comparison of two basal media, we selected one which gave the best colony growth and then sought to optimize the individual additives in the medium. Hydrocortisone, fetal bovine serum, and red blood cells all improved both plating efficiency and median size of colonies derived from breast carcinoma cells. Next, the concentrations of these three components were simultaneously idealized using response surface methodology. By these methods, we found that the optimal concentration of hydrocortisone was 0.35 μg/ml, fetal bovine serum was 6.5%, and red blood cells was 2.1 x 10^7 cells/ml. Using these culture conditions, we have achieved plating efficiencies of 0.39% and 0.19% for colonies with diameters greater than 50 (50 cells) or 70 (130 cells) μm, respectively.

INTRODUCTION

The unpredictable nature of the response of breast cancer to a particular therapeutic regimen is a major deterrent to optimization of effective treatment. For example, only half of the women treated with the combination cyclophosphamide, Adriamycin, and 5-fluorouracil respond with either partial or complete tumor regression (1). Advance knowledge of a patient's response to a specific therapy would be of obvious benefit to minimize unnecessary morbidity while providing predictably effective treatment. In vitro clonogenic assays using cells from individual human tumors have been developed with the aim of addressing this goal (2, 3). Currently, two cytotoxicity assay systems are being evaluated using human breast cancer cells. These include the anchorage-independent assay of Hamburger and Salmon (2) in which monodispersed breast carcinoma cells are cultured in an agar bilayer and an anchorage-dependent assay, adapted from methods developed by Stampfer et al. (4) for culturing human normal breast epithelium. The latter assay, while achieving reasonable plating efficiencies (5, 6), has typically used an initial culture period during which cell amplification and tumor cell subpopulation selection may occur. In addition, lymph node metastases cannot be grown by this method. Conversely, the anchorage-independent assay usually uses tumor cells immediately upon dissociation. However, low plating efficiency [0.009% reported by Sandbach et al. (7)] and other technical difficulties have limited the usefulness of this technique for breast cancer (7, 8). While plating efficiency is a major concern, an even more important limitation of the agar culture system may be its inability to support sufficient cell division for distinction between true colonies of viable cells and abortive colonies formed from reproductively dead cells.

Because of the aforementioned problems and the potential usefulness of a primary cell agar-based clonal cytotoxicity assay for breast carcinoma cells, we sought alternatives to the current methodology to improve the usefulness of the technique. In earlier studies, we investigated means to more efficiently produce monodispersed cells from solid breast carcinomas (9). In the current study, statistically motivated designs (10) are used to systematically define factors required for anchorage-independent growth of human breast carcinoma cells. These designs addressed specific problems that are associated with optimizing culture conditions for primary human tumor cells. For example, most individual tumors yield an insufficient number of cells to conduct a complete study of main effects and interactions of the many components present in tumor cell culture medium. In addition, pooling data from multiple individual tumors is not straightforward due to the heterogeneous distribution of genotypes and phenotypes present in their cell populations. These statistical problems are not unique to tumor cell tissue culture and have been successfully resolved by various approaches. Specifically, with partially confounded factorial designs (11), we have identified medium components that improve the in vitro growth of breast cancer cells. The optimized concentrations of these interacting components were estimated using response surface methodology (12). With these designs, we have improved both the plating efficiency and the average colony size for human breast carcinoma cells.

MATERIALS AND METHODS

Reagents. Collagenase, type III, and DNase I were obtained from Cooper Biomedical, Freehold, NJ. Hydrocortisone, cholaic acid, 17β-estradiol, progesterone, and triiodothyronine were purchased from Sigma Chemical Co., St. Louis, MO. Epidermal growth factor and insulin were obtained from Collaborative Research, Waltham, MA. The insulin used was low in zinc and noncytokytic. Bovine prolactin was provided by NIH, National Institute of Arthritis, Metabolism, and Digestive Diseases' National Pituitary Agency. Amphotericin B was purchased from Gibco, Grand Island, NY, and gentamicin was from the United States Biochemical Corp., Cleveland, OH. FBS was purchased from Sterile Systems, Inc., Logan, UT. Powdered α-MEM and Ham's F-12 medium were purchased from either Gibco, Grand Island, NY, or Flow Laboratories, McLean, VA. These media were prepared to 1X or 2X concentrations with distilled water that was shown by high-pressure liquid chromatography to be free of organic contaminants and buffered with HEPES (20 mM final concentration), Research Organics, Cleveland, OH.

Media. Two media formulations were initially evaluated. They are referred to as CSHM and HMM. CSHM consisted of α-MEM with FBS (10%), hydrocortisone (0.5 μg/ml), 17β-estradiol (5 ng/ml), insulin (5 μg/ml), progesterone (0.5 μg/ml), and prolactin (5 μg/ml) (9). HMM, which was formulated for clonal growth of anchorage-dependent human mammary cells (4), consisted of 30% Ham's F-12 medium. 30% α-MEM, and 40% conditioned medium with supplements of FBS (0.5%), insulin (10 μg/ml), hydrocortisone (0.1 μg/ml), 17β-estradiol (0.27 ng/ml), triiodothyronine (6.5 ng/ml), epidermal growth factor (5 ng/ml), and cholaic acid (1 μg/ml). The conditioned medium was a combination of medium conditioned by three cell strains: 20% human adult bladder cells (HS678B1); 10% human adult myoepithelial cells for breast carcinoma cells, we sought alternatives to the current methodology to improve the usefulness of the technique. In earlier studies, we investigated means to more efficiently produce monodispersed cells from solid breast carcinomas (9). In the current study, statistically motivated designs (10) are used to systematically define factors required for anchorage-independent growth of human breast carcinoma cells. These designs addressed specific problems that are associated with optimizing culture conditions for primary human tumor cells. For example, most individual tumors yield an insufficient number of cells to conduct a complete study of main effects and interactions of the many components present in tumor cell culture medium. In addition, pooling data from multiple individual tumors is not straightforward due to the heterogeneous distribution of genotypes and phenotypes present in their cell populations. These statistical problems are not unique to tumor cell tissue culture and have been successfully resolved by various approaches. Specifically, with partially confounded factorial designs (11), we have identified medium components that improve the in vitro growth of breast cancer cells. The optimized concentrations of these interacting components were estimated using response surface methodology (12). With these designs, we have improved both the plating efficiency and the average colony size for human breast carcinoma cells.
Tumors. Primary breast carcinomas were obtained from local Madison, WI, hospitals (University Hospital and Clinics, Madison General Hospital, St. Mary's Hospital, and Methodist Hospital) following wide excisional biopsies or mastectomies. All specimens were collected in α-MEM containing FBS (10%), gentamycin (50 μg/ml), and amphotericin B (0.5 μg/ml) and transported on ice to our tissue culture laboratories within 1 h of surgery. The tissue was then trimmed to remove any fat or grossly necrotic regions. Representative portions were removed to verify tumor histopathology. Only diagnosed primary breast carcinomas were used in this study.

Tumor Disaggregation. Breast carcinomas were dissociated by previously evaluated methods (9). Specifically, tumors were scalar minced into approximately 1-mm pieces and then suspended in a solution of collagenase type III (2 mg/ml) dissolved in α-MEM containing FBS (5%), gentamycin (50 μg/ml), and amphotericin B (0.5 μg/ml). This suspension was incubated for 20-24 h at 37°C on a rotary platform (200 rpm). Following this digestion period, 3 ml of DNase I (0.05%) was added to each 100 ml of tumor suspension and incubated for an additional 10 min. The tumor suspension was washed twice with α-MEM, resuspended, and gravity filtered through a 53-μm nylon mesh filter (Tetko, Elmsford, NY). The monodispersed cells were counted, and the ability of the cells to exclude trypan blue dye was estimated.

Agar Culture. Primary breast carcinoma cells were cultured in gridded 35-mm tissue culture dishes (Lux Scientific Co., New York, NY) using a modified agar bilayer technique (2). A 1% concentration of Bactoagar (Difco Laboratories, Detroit, MI) was prepared by dissolving the agar in heated double-distilled water. The base layer of the bilayer consisted of a 1:1 mixture of 1% agar and a 2× concentration of α-MEM medium. The upper layer, containing 106 trypan blue dye-excluding tumors, had a final agar-medium concentration of 0.3% prepared by combining 30% 1% agar, 20% of water, and 50% of 2× α-MEM medium. After the cells were seeded, the cultures were allowed to solidify at room temperature, examined for any presence of cell clumping, and incubated at 37°C. Clonal growth was evaluated on Day 14. Colony diameters were measured using a calibrated objective micrometer with an inverted phase microscope.

RBC Source and Preparation. CF-1 mice, Fischer 344 rats, and Sprague-Dawley rats were purchased from Harlan Sprague-Dawley, Madison, WI. August rats were originally obtained from Shaw's Farm, Blackthorn, Bicester, Oxford, England, and then bred in our laboratory. Blood samples were obtained, using a heparinized syringe, from the abdominal aorta in rats, the heart in mice, and the cuboidal fossae in humans. For all species, an equal amount of blood from male and female donors was pooled. Whole blood was centrifuged at 3000 x g for 15 min, the plasma with buffy coat was removed, and the packed RBC were washed 3 times in 0.85% phosphate-buffered saline and resuspended to the original blood volume. The RBC suspension was counted and checked for contamination with WBC and platelets. Following Courtenay’s protocol (13), a 1:40 dilution of RBC was initially used as a medium supplement. This dilution provided 7.0 x 109 cells/ml. RBC suspensions were stored at 4°C for 7 days before use and only used until 14 days postcollection.

Modified RBC. Resealed RBC membranes (ghosts) were prepared by modified methods of Wood and Passow (14). A sterile 12- x 1.5-in water-jacketed chromatography (LKB Instruments, Inc., Rockville, MD) was prepared in a laminar flow hood and connected to a refrigerated (0°C) circulating water bath. The column was filled with 120 ml of sterilized Bio-Gel A-50 (50–100 mesh) (BioRad, Richmond, CA) and flushed with three bed volumes of a 0.1 mM EDTA solution buffered to pH 6.0 with 15 mM HEPES (Solution A) which subsequently formed Zone 1. Zone 2 was formed by switching to double-distilled water buffered to pH 7.6 with 20 mM HEPES (Solution B), and elution was continued until a volume equal to 40% of the bed volume had been applied. Zone 3 was formed by switching to a 146 mM NaCl solution buffered to pH 7.6 with 20 mM HEPES (Solution C), and elution was continued until a volume equal to 10% of the bed volume had been applied. Once the column had been equilibrated as described, the packed RBC were resuspended to 10% cell density in Solution C (22°C). A volume of this solution equal to 10% bed volume was added to the column. Elution was continued at 0°C with Solution C. Unsealed ghosts were collected from effluent in a vessel immersed in an ice bath. Ghosts were resealed by adding α-MEM and incubating at 0°C for 5–10 min and then at 37°C for 45 min. Immediately after collection, an aliquot of ghosts was removed, trypan blue dye was added to the suspension, and then the ghosts were resealed. The aliquot was examined for the presence of blue ghosts to indicate that the resealing had occurred, and the supernatant of dyed ghosts was tested with a spectrophotometer for absence of dye to indicate that the ghosts had tightly sealed. Ghosts were shown to have tightly resealed in the preparations. Ghosts were prepared weekly and stored at 4°C.

To prepare lysed RBC, packed RBC were resuspended in a hypotonic solution of double-distilled water buffered to pH 7.6 with 20 mM HEPES. The mixture was agitated on a wrist action shaker for 1 h and then stored overnight at 4°C. An aliquot was examined after 24 h to ensure the absence of intact RBC. The soluble contents of RBC were separated from the membrane cell fraction by centrifugation (13,000 x g, 30 s). The soluble fraction, present in the supernatant, was assayed for hemoglobin content using the cyanmethemoglobin method (15). All RBC components were added to the medium in the amount found in 2.1 x 105 RBC/ml.

Oxygen Concentration. Cultures were maintained in an atmosphere containing 5% CO2, 5% or 20% O2, and the remaining N2. To maintain a 20% O2 (148 PO2) concentration, the cultures were incubated in a 5% CO2 and 95% air. A 5% O2 (37 PO2) concentration was maintained using a triple gas processor incubator (Forma Scientific, Marietta, OH). Oxygen levels were routinely monitored using a blood gas analyzer (Instrumentation Laboratory, Inc., Lexington, MA).

Immunohistochemistry. Our agar bilayer system was modified by substituting methyl cellulose for agar in the top layer as described by Buick et al. (16).The use of methyl cellulose allowed the recovery of intact colonies. These colonies were fixed, embedded in paraffin, cut, and stained. Samples of the original tumor material were processed similarly. Immunohistochemical staining was performed by exposing sections to primary antibody, followed by a biotinylated secondary antibody. This primary-secondary complex was then exposed to a preformed avidin and biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) followed by a peroxidase substrate solution. The primary antibodies used in these studies included one polyclonal antibody specific for skin keratin (6) [Accurate Chemical Co., Westbury, NY; (b) locally prepared], one against carcino-embryonic antigen (Accurate Chemical Co., Westbury, NY) and one monoclonal against milk fat globule antigen (Accurate Chemical Co., Westbury, NY). In all experiments both positive and negative controls were run in parallel.

Normal Human Cell Culture. Normal human mammary epithelial cells, obtained from residual surgical tissue recovered from healthy premenopausal women undergoing reduction mammoplasties, were cultured by methods developed by Stampfer et al. (4) and modified by us (17). Mammary epithelial cell strains (A491, A676) grew well in both mass culture and clonal culture on plastic substrates. Primary cell cultures were grown to approximately 75% confluence, trypsinized, and seeded in an agar culture of either CSHM or our improved agar medium containing hydrocortisone (0.35 Mg/ml), FBS (6.5%), and RBC (2.1 x 107 cells/ml). Low-passage human embryo skin diploid fibroblasts (strain S132), human mammary tumor fibroblasts (strain B543), and mammary epithelial cell strains (A491, A676) grew well in our improved agar medium with 15% FBS and prepared for agar culture as described above.

Statistically Motivated Designs. Optimization of the in vitro growth of cells from tumors requires statistically motivated experimental designs. These designs make efficient use of the limited number of cells that can be obtained from each tumor and also avoid confounding results due to tumor-to-tumor heterogeneity. Specifically, two types of designs were used in this investigation. The first design was used to address qualitative questions, i.e., should a specific culture additive or...
condition be used? This type of design is referred to as a factorial design. The second type of design used is one that addresses quantitative questions, i.e., how much of an additive should be used for optimal results. This type of design is referred to as a response surface design. The first factorial design used was the $2^2$ partially confounded factorial design (10, 11). This design allowed for the simultaneous investigation of five factors (hydrocortisone, 17β-estradiol, insulin, progesterone, and prolactin). In this way, information was available on the effect of each hormone on plating efficiency and cell growth. Moreover, this experiment also allowed one to check for possible interactions among these hormones. The statistical technique of partial confounding was used to reduce the total amount of tumor material required to examine the main effects and the higher order interactions of these hormones. The form of the response surface (i.e., a mathematical expression describing how the plating efficiency varied with different combinations of hydrocortisone, FBS, and RBC) was developed using least-squares methodology. The fit of these models was assessed using the $F$ test (10, 11).

Response surface methodology (10–12) was used to optimize three additives (hydrocortisone, FBS, RBC) that were qualitatively found to be growth promoting in the factorial experiments. In particular, the response surface methodology prescribed an iterative approach for this optimization. An initial experiment was performed which examined the effects of these additives over a wide range of settings. Two further response surface experiments were then used to locate the optimal concentrations of the additives. The form of the response surface (i.e., a mathematical expression describing how the plating efficiency varied with different combinations of hydrocortisone, FBS, and RBC) was developed using least-squares methodology. The fit of these models was again tested using the $F$ test (10–12).

RESULTS

Initial Media Comparisons. CSHM and HMM were compared in an initial attempt to determine the general medium requirements for clonal growth of primary breast carcinoma cells in vitro. Each medium was assessed for its ability to support clonal growth. The end points examined on Day 14 were plate efficiency and cell growth. Moreover, this experiment also allowed one to check for possible interactions among these hormones. The statistical technique of partial confounding was used to reduce the total amount of tumor material required to examine the main effects and the higher order interactions of these hormones (10, 11).

A second factorial design, a $4 \times 2^2$ partially confounded factorial design, was used to corroborate the results of the $2^2$ factorial experiment, as well as further explore the effects of oxygen, RBC, and their interactions on cell growth. As in the previous factorial experiment, the statistical technique called partial confounding was used to reduce the total amount of tumor material required for the experiment. Furthermore, in both of these factorial experiments, the significance of main effects and high order interactions was assessed by examining the parameters of a regression model which was specified by the particular design. The method of least-squares was used to obtain parameter estimates and standard errors (10, 11). Significance was assessed using the $F$ test (10, 11).

Table 1 An initial comparison of two media for the support of clonal growth of primary human breast carcinoma cells in agar culture

<table>
<thead>
<tr>
<th></th>
<th>Mean % of plating efficiency with colony diameter of 50 μm</th>
<th>Mean colony diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;50 μm</td>
<td>&gt;70 μm</td>
</tr>
<tr>
<td>CSHM</td>
<td>0.197**</td>
<td>0.129*</td>
</tr>
<tr>
<td>HMM</td>
<td>0.173*</td>
<td>0.096*</td>
</tr>
<tr>
<td>CSHM without hormones</td>
<td>0.096**</td>
<td>0.024**</td>
</tr>
<tr>
<td>CSHM without FBS</td>
<td>0.016</td>
<td>0.0001</td>
</tr>
<tr>
<td>CSHM without FBS or hormones</td>
<td>0.004</td>
<td>0.0000***</td>
</tr>
</tbody>
</table>

* Within each column, like-starred data did not differ significantly; all others differ at the 0.05 level when tested by an analysis of variance procedure.

RESULTS

Hormonal Requirements. The hormonal supplements in CSHM consisted of hydrocortisone, 17β-estradiol, insulin, progesterone, and prolactin. A systematic examination of the main effects and interactions of these hormones on the development of primary human breast carcinoma colonies in an agar assay was conducted using a $2^2$ partially confounded factorial design (11) (Table 2). This design was chosen because limited numbers of cells available made it impossible to run a complete factorial design for each tumor. In addition, this design avoided confusing hormonal effects and interactions with intertumor heterogeneity. The end points examined were colonies with diameters greater than 50 μm, colonies with diameters greater than 70 μm, and average colony diameter.

Of all the hormones evaluated for their ability to promote the clonal growth of breast tumor cells, only hydrocortisone was found to enhance clonal growth irrespective of any other hormonal supplementation ($P < 0.001$, for all end points). 17β-Estradiol, prolactin, and progesterone had no main effects ($P > 0.25$). Insulin was found to inhibit clonal formation ($P < 0.001$, for all end points). A further examination of the data indicated a significant two-way interaction between insulin and 17β-estradiol ($P < 0.05$, for all end points). While the overall effect of insulin was to suppress clonal growth, Fig. 1 illustrates that, in the presence of insulin, the effect of 17β-estradiol was to promote growth. In the absence of insulin, there appears to be no effect due to 17β-estradiol. This was true for the colony end points at both 50 and 70 μm.

RBC and Oxygen. Based on the findings of Courtney (13), we investigated the effect of the combination of a 5% atmospheric oxygen growth environment and the addition of RBC from August rats to the culture medium to determine their effects on clonal growth of human tumor cells. The goal of the experiment presented in this section was to examine the effects and interactions of these two variables in the agar-based culture, in the presence of various hormone combinations.

As in the previous section, a complete factorial experiment could not be run due to limited tumor material. For this reason, a $4 \times 2^2$ partially confounded factorial design was used (Table 3). The four hormone combinations tested were hydrocortisone (0.5 μg/ml), hydrocortisone (0.5 μg/ml) plus epidermal growth factor (5 ng/ml), hydrocortisone (0.5 μg/ml) plus insulin (5 μg/ml), and hydrocortisone (0.5 μg/ml) plus prolactin (5 μg/ml). Atmospheric oxygen was tested at concentrations of either 5% or 20%. RBC were either present or absent. Again, end points used were colonies with diameters in excess of 50 and 70 μm and average colony size. The results confirmed the growth-stimulatory effects of hydrocortisone and the growth-inhibitory effects of insulin. The addition of epidermal growth factor to hydrocortisone-containing medium did not significantly alter growth. A strong positive interaction was found between 20% atmospheric oxygen concentration and RBC supplementation in the culture medium for all end points ($P < 0.001$). However, in the absence of RBC, the atmospheric oxygen concentration had little effect. Conversely, the addition of RBC had no effect at a 5% atmospheric oxygen concentration (Fig. 2).

Because of the importance of RBC interactions described above, we wished to further investigate their role in stimulating growth. Since it has been reported (13) that RBC from August rats were unique in their ability to stimulate tumor cell clonal growth, we compared RBC from August rats to RBC of other...
Table 2 A 24 partially confounded factorial design and results used to assess the effects of five hormones on the growth of primary human breast carcinoma cells in culture

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>None, 0.014</td>
<td>BC, 0.013 ABD, 0.192 ACD, 0.127 ABE, 0.186 ACE, 0.124 DE, 0.019 BCDE, 0.035</td>
</tr>
<tr>
<td>AB, 0.022</td>
<td>AC, 0.000 D, 0.016 BCD, 0.000 E, 0.000 BCE, 0.000 ABDE, 0.012 ACDE, 0.000</td>
</tr>
<tr>
<td>A, 0.165</td>
<td>ABC, 0.121 BD, 0.033 BE, 0.019 CE, 0.007 ADE, 0.093 ABCDE, 0.075 CD, 0.005</td>
</tr>
<tr>
<td>B, 0.007</td>
<td>C, 0.023 AD, 0.337 ABD, 0.222 AE, 0.370 ABCE, 0.210 BCE, 0.032 CDE, 0.028</td>
</tr>
<tr>
<td>None, 0.060</td>
<td>AD, 0.157 ABC, 0.075 BCD, 0.000 ABE, 0.170 BDE, 0.009 CE, 0.000 ACDE, 0.161</td>
</tr>
<tr>
<td>AB, 0.316</td>
<td>BD, 0.137 C, 0.035 ACD, 0.160 E, 0.215 ADE, 0.217 ABCE, 0.161 BCDE, 0.052</td>
</tr>
<tr>
<td>A, 0.190</td>
<td>D, 0.019 BC, 0.004 ABCD, 0.089 BE, 0.007 ABDE, 0.096 ACE, 0.004 CDE, 0.000</td>
</tr>
<tr>
<td>B, 0.019</td>
<td>ABD, 0.336 AC, 0.026 CD, 0.000 AE, 0.357 DE, 0.022 BCE, 0.000 ABCDE, 0.331</td>
</tr>
</tbody>
</table>

* Tumors were obtained from eight women with infiltrating carcinomas.
* The capital letter(s) in each group denotes the hormone(s) used in each treatment group, where A is cortisol (0.5 μg/ml); B is 17β-estradiol (5 ng/ml); C is insulin (5 μg/ml); D is progesterone (0.5 μg/ml); and E is prolactin (5 μg/ml).
* The high-order interactions of ABC, ADE, and BCDE are partially confounded in Tumors 1-4, while the interactions ABD, BCE, and ACDE are partially confounded in Tumors 5-8.

Table 3 A 4 x 24 confounded factorial design and results used to investigate the effects of RBC, atmospheric oxygen concentrations, and hormones on the growth of primary human breast carcinoma cells in culture

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>000, 0.142</td>
<td>011, 0.400 100, 0.086 111, 0.366 201, 0.044 210, 0.067 301, 0.046 310, 0.049</td>
</tr>
<tr>
<td>001, 0.092</td>
<td>010, 0.042 101, 0.118 110, 0.029 200, 0.047 211, 0.220 300, 0.030 311, 0.154</td>
</tr>
<tr>
<td>000, 0.089</td>
<td>011, 0.433 101, 0.174 110, 0.044 200, 0.069 210, 0.093 300, 0.089 311, 0.408</td>
</tr>
<tr>
<td>001, 0.347</td>
<td>010, 0.174 100, 0.150 111, 0.504 200, 0.104 211, 0.383 301, 0.158 310, 0.049</td>
</tr>
<tr>
<td>000, 0.084</td>
<td>011, 0.287 101, 0.237 110, 0.023 200, 0.063 211, 0.107 301, 0.057 310, 0.049</td>
</tr>
<tr>
<td>001, 0.121</td>
<td>010, 0.044 100, 0.334 111, 0.661 201, 0.163 210, 0.086 300, 0.044 311, 0.358</td>
</tr>
</tbody>
</table>

* Tumors were obtained from six women with infiltrating carcinomas.
* Each group is described by three digits. The first represents the hormone combination, the second represents the level of RBC, and the third represents the level of atmospheric oxygen in a given group, where: first digit 0, cortisol (0.5 μg/ml); 1, cortisol (0.5 μg/ml) plus epidermal growth factor (5 ng/ml); 2, cortisol (0.5 μg/ml) plus insulin (5 μg/ml); 3, cortisol (0.5 μg/ml) plus prolactin (5 μg/ml); second digit 0, RBC absent; 1, RBC present (7 x 10⁷ cells/ml); and third digit 0, 5% atmospheric oxygen concentration; 1, 20% atmospheric oxygen concentration.

* In all cases, the numbers represent the plating efficiencies for colonies with diameter in excess of 70 μm.
tumors serving as the blocks and the four treatments being 0.5% FBS, 2.5% FBS, 5.0% FBS, and 10% FBS. The culture medium used was CSHM with varying levels of FBS as indicated. For all end points examined, 10% FBS was superior to any other concentration tested. The second experiment was run as a 2 × 8 randomized block design with the blocks being the two tumors and the treatments being CSHM with hydrocortisone at the following concentrations: 0.0 μg/ml; 0.1 μg/ml; 0.3 μg/ml; 0.5 μg/ml; 0.7 μg/ml; 1.0 μg/ml; 2.0 μg/ml; and 5.0 μg/ml. We found the greatest stimulatory effect at hydrocortisone concentrations between 0.5 μg/ml and 0.7 μg/ml. To examine the effects of escalating the concentration of RBC in culture medium, a 6 × 4 randomized complete block experiment was conducted. In each of six tumors, the four levels of RBC tested were: no RBC; 1.75 × 10^7 cells/ml; 7 × 10^7 cells/ml; and 2.8 × 10^8 cells/ml. Using the 95% Bonferroni analysis for simultaneous pairwise contrasts, for each of the three end points examined, clonal growth in medium containing between 1.75 × 10^7 cells/ml and 7.0 × 10^7 cells/ml was found to be best.

The goal of the final series of experiments was to optimize plating efficiency and colony size by selecting the appropriate concentrations of hydrocortisone, FBS, and RBC. These experiments differed from the previous series in that the three medium components were optimized simultaneously to ensure that the results would reflect their unique interaction. Using response surface methodology, a series of three sequential experiments were performed to locate and define regions of optimized performance. The first experiment explored the nature of the response surface over a large region (Fig. 3A, Box I). It should be noted that the central starting point in this initial response surface analysis was provided by data from the previous preliminary series of experiments. Based on the results of the initial response surface exploration, two smaller regions were examined: one region centrally located within the first region (Fig. 3A, Box II) and the second located toward the lower left (Fig. 3A, Box III).

These experiments were conducted in blocks because tumor size limited the numbers of cells available for each study. For this reason, it was essential that the blocks be allocated to tumors in such a manner to avoid confusing the effects being investigated with any differences between tumors. The orthogonally blocked central composite design allows for an efficient use of tumor material, while ensuring that tumor-to-tumor heterogeneity effects are accounted for prior to estimating the optimal concentrations of tested components. Fig. 3B illustrates the orthogonally blocked central composite design used for each of the three experiments, and the corresponding settings of hydrocortisone, FBS, and RBC for the three experiments are given in Table 6.

An application of the second-order model to the results of the first experiment (centered at hydrocortisone, 0.5 μg/ml, 10% FBS, 3.5 × 10^7 RBC/ml; Fig. 3A, Box I) revealed strong evidence of lack of fit (P < 0.005). In other words, a simple quadratic surface provided a poor model for the true surface under the wide range of concentrations explored in the first experiment. For this reason, a second experiment (centered at

![Graph](image-url)
hydrocortisone, 0.5 µg/ml, 10% FBS, 3.5 x 10^7 RBC/ml; Fig. 3A, Box II) covering a more limited area was run within this region. However, even within this contracted range, there was again strong evidence that a quadratic model could not adequately describe the response surface (P < 0.025).

An examination of the data from the first two experiments revealed that the lower left region of the initial region (Fig. 3A, Box I) had improved plating efficiencies and colony diameters. Therefore, another orthogonally blocked central composite design (centered at hydrocortisone, 0.25 µg/ml, 6% FBS, and 1.4 x 10^7 RBC/ml; Fig. 3A, Box III) was used to explore this region. For this choice of settings, there was little evidence of lack of fit for the second-order model for any of the three clonal endpoints (P > 0.10).

Based on this second-order model, the maximum plating efficiency for colonies with diameters greater than 70 µm was located at concentrations of hydrocortisone, 0.35 µg/ml, 6.5% FBS, and 2.1 x 10^7 RBC/ml. The second-order model used to obtain these optimal values is given by the following equation:

\[ 12.23 + 1.23x + 2.21y + 1.73z - 1.51xy + 0.33xz - 0.29yz - 0.72x^2 - 1.37y^2 - 0.92z^2 \]

where \( x \) is \([\text{hydrocortisone}] - 0.25\), \( y \) is \([\text{FBS}] - 6.0\), and \( z \) is \([\text{RBC}] - 1.4 \times 10^7\). The graphical representation of Equation A is presented in Fig. 4. This surface response graph illustrates that any combination of the three agents tested, which lie on the surface of the ellipsoid, yields equal growth stimulation of primary breast cancer cells in an agar-based clonal culture system.

Thus far, we evaluated 12 primary tumors using our optimized dissociation method (9) and improved medium outlined above. We have achieved a mean plating efficiency of 0.39 ± 0.05% and median plating efficiency of 0.33% for colonies with diameters in excess of 50 µm. For the 70-µm end point, the mean plating efficiency was 0.19 ± 0.03% and median plating efficiency of 0.15%. In addition, two chest wall recurrent tumors gave plating efficiencies of 0.52% and 0.79% (50 µm), and a cervical lymph node metastasis gave a 50-µm colony-plating efficiency of 0.52%. It should be noted that colonies from this latter tumor were examined by electron microscopy and found to be composed of epithelial cells.

Normal human mammary epithelial cells did not form colonies in agar culture. Small cell clusters were occasionally seen. Specifically, in our starting medium CSHM, mammary epithelial strain A491 had a plating efficiency of 0.15% for colonies with diameters in excess of 70 µm. The intersection of the three axes in the center of the ellipsoid represents the point of maximum plating efficiency. This point corresponds to the concentrations: hydrocortisone, 0.35 µg/ml; FBS, 6.5%; and RBC, 2.1 x 10^7 cells/ml.

Normal human mammary epithelial cells did not form colonies in agar culture. Small cell clusters were occasionally seen. Specifically, in our starting medium CSHM, mammary epithelial strain A491 had a plating efficiency of 0.005% for colonies with diameters greater than 50 µm and 0.00% for colonies with diameters greater than 70 µm. When cells of the A491 strain were grown in our final improved medium, no colonies over 50 µm were seen in either of these media. Cells used in the above assays were from primary cultures of normal mammary cells, i.e., secondary cell clonal assay. In general, the tumor cell clonal agar data presented here are for primary cells, i.e., one passage earlier than normal breast cells. Thus to control for this clonal difference, six breast tumors were grown in primary culture on plastic by identical methods.
used for normal breast cells. They were then trypsinized and plated either onto plastic or in agar. Positive clonal growth was obtained for all six tumors both on plastic (average efficiency, 3.0%) and in agar (average efficiency, 0.2%). We also tested three fibroblast strains for growth in our agar system. No growth was obtained in agar for any of these cells. However, when the two breast (normal and malignant)-derived fibroblasts were tested for clonal growth on plastic at the same passage level, positive results were obtained. Strain 4488F (normal) had a plating efficiency of 12%, and B543 had a plating efficiency of 8.6%.

Sections from two randomly selected tumors and anchorage-independent colonies grown from these tumors were stained for “skin-like” keratins, carcinomaembryonic antigens, and milk fat globule antigen. The epithelial components of both tumors stained positive for these antigens in a heterogeneous manner, i.e., not all cells stained. The stromal cells in these tumor sections did not stain with any primary antibody used. Heterogeneous staining was also found in the tumor cell colonies (Table 7).

DISCUSSION

We have systematically explored nutrient and environmental requirements for anchorage-independent clonal growth of human breast carcinoma cells in an agar bilayer system with the goal of improving plating efficiency, colony size, and reliability. Initially, we compared HMM (4) and CSHM (9). While HMM was superior to CSHM in its support of anchorage-dependent growth of human breast carcinoma cells, in an anchorage-independent agar-based assay, we found no difference between these media. Because CSHM was better defined and easier to prepare, this medium was used as the foundation for defining a breast carcinoma-specific medium for the support of anchorage-independent growth. CSHM contained hydrocortisone, 17β-estradiol, insulin, prolactin, and progesterone, each having a physiological role in mammary gland growth and development. Of these five hormones, only hydrocortisone was growth stimulatory, while insulin was growth inhibitory. It must be stressed that these hormones were tested in the presence of 10% FBS, which itself provides subphysiological levels of each hormone examined. Thus, we do not exclude an absolute requirement for these hormones. Furthermore, the hormones shown to be ineffective in our mammary cell culture system were only tested at a single concentration. Even though the concentrations tested were shown to be effective in other in vitro systems, it is possible that they would be effective in our system at alternative concentrations.

At this stage, our now simplified medium contained only FBS and hydrocortisone as growth-stimulatory additives. In order to further improve clonal growth, we explored the use of a low atmospheric oxygen concentration in conjunction with August rat RBC. In our system, human breast carcinoma cells did not respond to these factors in the manner reported for other tumor types by Courtney and coworkers (3, 13). We found cultures grown in the presence of 5% atmospheric oxygen and RBC did not grow better than cells grown in our medium without RBC in a 20% atmospheric oxygen concentration. However, by using a factorial design, we were able to detect a two-way interaction between oxygen and RBC. The addition of RBC to cell cultures increased both plating efficiency and colony diameter only in the presence of a 20% atmospheric concentration of oxygen. In a follow-up experiment, we found that August rat RBC were not unique when compared to RBC from other rat strains, as suggested by Courtney (13). Moreover, human RBC were equally as effective as rat RBC, although mouse RBC did not stimulate growth. An exploration of the RBC subcellular fractions identified growth-stimulatory properties in both membranous and nonmembranous components of the RBC. Further investigation will be required to better define the growth-stimulatory factors found in RBC.

After demonstrating growth-stimulatory properties of hydrocortisone, FBS and RBC in our culture system, we used response surface methodology, developed by Box and Wilson (12), to simultaneously optimize the concentrations of these three components. This design uses the method of steepest ascent to locate the maximum plating efficiency and then approximate the surface in the vicinity of this maximum with a fitted second degree equation. In other words, the results at each stage are used to guide the level of treatments at the next stage, and each stage in the experiment requires only a limited number of runs to ensure efficiency. We found the ideal concentration of these components to be: hydrocortisone, 0.35 μg/ml; FBS, 6.5%; and RBC, 2.1 × 10^7 cells/ml. Additionally, it should be stressed that, if these three components were idealized separately, thereby ignoring their interactions, less effective concentrations would have been obtained.

Thus far, 12 human breast carcinomas have been dissociated and cultured using our optimized conditions. We have achieved a mean plating efficiency of 0.39 ± 0.053% and a median plating efficiency of 0.33%. This represents over a 35-fold increase in the median plating efficiency of 0.009% (7) for human breast carcinoma cells dissociated and assayed using the standard anchorage-independent agar bilayer assay of Hamberger and Salmon (2).

While almost all tumors grew well with our improved culture conditions, we still see a heterogeneous quantitative response between tumors. This suggests that, while our culture system is good for the “average” breast carcinoma, it may not be ideal for each individual tumor. Future investigation will be directed to identify breast carcinoma subtypes which may require modification in our culture system.

We have previously demonstrated the epithelial nature of the agar colonies by electron microscopy (9). Here we have shown that both normal human fibroblasts from skin, normal breast, and breast tumor as well as breast epithelial cells do not form colonies in our agar culture system, while cells from primary and metastatic breast tumors did form colonies. In addition, we find that, when stained for three epithelial markers, our colonies show a heterogeneous staining pattern similar to that seen in the tumor of origin. Thus, we feel our agar culture system supports the growth of breast tumor cells and does not support normal fibroblasts or breast epithelial cells. It should, however, be mentioned that, while it is generally thought that normal cells require anchorage for growth, there have been several reports in which both presumably normal fibroblasts (20, 21) and epithelial cells (22) have been shown to grow in an anchorage-independent manner. For example, Peehl and Stanbridge

Table 7 Immunohistochemical colony staining

<table>
<thead>
<tr>
<th>Antibody*</th>
<th>Tumor B543</th>
<th>Tumor B677</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-keratin a</td>
<td>38</td>
<td>44</td>
</tr>
<tr>
<td>Anti-keratin b</td>
<td>19</td>
<td>46</td>
</tr>
<tr>
<td>Anticarcinoembryonic antigen</td>
<td>100</td>
<td>92</td>
</tr>
<tr>
<td>Anti-milk fat globule membrane</td>
<td>65</td>
<td>12</td>
</tr>
</tbody>
</table>

* See “Materials and Methods” for details.
(20) demonstrated anchorage-independent growth of normal human fibroblasts using medium containing high levels of serum and hydrocortisone. It should be noted that they (20) reported that a lower layer of Difco agar, as we use here, completely eliminated the growth of fibroblasts in the upper layer. Azzarone et al. (21) recently reported that skin fibroblasts from the skin of breast cancer patients grew in anchorage-independent culture. However, their criteria for growth were the presence of colonies of only 30 cells. Stamper et al. (22) have reported data agreeing with our findings that normal breast epithelial cells do not grow in a semisolid medium. However, they (22) also reported growth of breast epithelial cells obtained from the periphery of breast carcinomas. This growth in Methocel might represent the growth of malignant cells that were distant from the main tumor in that Holland et al. (23) report finding distant cancer cells in 60% of breast cancer cases. This percentage is conservative in that only large groups of cancer cells could be detected by the routine pathology methods used.

Finally, we have recently successfully used this assay with cells from five patients to generate a radiation dose-cell survival curve (18) which we and others (24) view as a critical test of a true clonal assay. We feel that this clonal assay system is now ready for clinical evaluation.

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Systematic Optimization of the Clonal Growth of Human Primary Breast Carcinoma Cells

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