STUDY OF PHARMACEUTICAL INDUSTRIAL PROBLEMS

Final Report
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By: Jack H. Pincus, Ph.D.

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NASA LYNDON B. JOHNSON SPACE CENTER
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Approved by:

W. A. Skinner, Executive Director
Life Sciences Division

SRI International

333 Ravenswood Ave. • Menlo Park, California 94025
(415) 326-6200 • Cable: SRI INTL MPK • TWX: 910-373-1246

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INTRODUCTION

Tumor antigens are proteins that are detectable by the host's immune system and that appear on the surface of tumor cells but are not present on normal cells.[1] Some of these give rise to an immune response in a tumor-bearing host that is strong enough to augment the host's specific immunity to the tumor, thereby allowing the immune system to destroy it.[2,3] These antigens can be used therapeutically (immunotherapy). In addition, some tumor antigens are released from the tumor cell surface and circulate in the blood. Immunoassays using antibodies against these antigens can measure them in serum and can be used for mass screening of populations for early diagnosis of cancer (immunodiagnosis).[4,5] Purified tumor antigens are needed for immunotherapy and for producing antibodies for use in immunodiagnosis. Therefore these tumor cell membrane products are useful as pharmaceuticals.

A glycoprotein antigen present on the surface of human colon carcinoma cells has been termed the carcinoembryonic antigen (CEA).[6] It has been purified and a radioimmunoassay has been developed to detect it in serum. A kit containing purified CEA and other reagents for performing this assay is sold by Hoffman-La Roche, Inc. (Nutley, New Jersey), and is used by clinicians to measure CEA levels in colon cancer patients at different times after surgery as an aid in evaluating the patient's prognosis.

CEA is purified from hepatic metastases of colon carcinoma obtained at surgery. To obtain a tissue mass containing a large amount of CEA, many individual tumor specimens must be pooled; this can be cumbersome and expensive. In addition, each of the specimens may be at a different stage of growth and contain variable amounts of CEA. Therefore, pooled tumor tissue obtained from random surgical cases may not always yield the same amount of antigen. Moreover, tumors that are in an advanced stage of growth often contain a large mass of necrotic tissue and only a small percentage of the specimen is usable.

An alternative to using whole tumor tissue is to use tumor cells grown in tissue culture. Tissue culture techniques permit the growth, under defined conditions, of a relatively homogeneous cell population that has high viability. In addition, growth conditions can be found under which optimal amounts of tumor antigen are always produced by the cells. Mass culture techniques have been developed for growing mammalian cells (usually lymphoblastoid cells) in suspension in quantities large enough for the isolation of tumor antigens.[7] However, most cultured cells must adhere to a surface to grow, and growing large numbers of adherent cells requires a large surface area and large amounts of medium.

Recently, we performed a study for a commercial client in which we identified pharmaceutical products that can only be produced by cells in culture and anlyzed the economic limitations to the production of these substances. A major limitation is the cost of growing large numbers of adherent cells in monolayer culture. We concluded that methods that provide for a large surface area in a small volume will overcome this limitation.

An apparatus has been developed that uses a spiral plastic sheet in a plastic container.[8] This increases the surface area per unit volume and reduces the amount of medium needed per unit surface area. However, with this apparatus, a large amount of culture medium relative to the number of cells obtained is still needed. Therefore, the method is not suitable for producing cells economically.

Another approach to producing adherent mammalian cells on a large surface area in a small volume involves the use of ultrafiltration capillaries packed into a small chamber.[9] The medium is pumped through the capillaries, thereby feeding nutrients to the cells. Unfortunately, the apparatus has not been scaled up sufficiently for the large-scale commerical production of cells.

The growth of cells on microspheres in a vessel of suitable size may overcome some of the difficulties associated with the large-scale production of adherent cells. Microspheres provide a larger surface area for cell anchorage per unit volume of culture medium than does a flat surface or capillaries. In addition, with microspheres, adherent cells can be grown in spinner culture, thereby facilitating nutrient utilization and removal of waste products. However, many microspheres must be stirred vigorously to remain in suspension, and this can have an adverse effect on cell viability and growth.

The growth of cells on microspheres in a weightless environment may overcome this difficulty. The position of the spheres suspended in a liquid culture medium would be stable and the culture would require only minimal agitation to ensure adequate availability of nutrients and removal of waste products. In addition, the weightless environment could alter cell-to-cell interaction of other physiological properties, perhaps resulting in greater yields of CEA per unit cell mass.

In the research described in this report, we evaluated the growth of a human colon carcinoma cell line (SK-CO-1) and its production of CEA in monolayer culture and on single layers of glass beads in unit gravity. In addition, we identified the limitations of using a microsphere-cell growth system in unit gravity and considered how these may be overcome in space.

PROGRESS REPORT

Task 1 - Growth of Cultured Human Colon Carcinoma Cells on a Monolayer and CEA Production

We used the human colon carcinoma cell line SK-CO-1 for all experiments described in this report. This cell line was initiated in 1972 by Drs. G. Trempe and L. J. Old at the Sloan Kettering Institute for Cancer Research, New York, New York, from an adenocarcinoma of the colon obtained from a 65-year-old male. The establishment of this line and its karyotype, morphology, and susceptibility to viruses have been described by Fogh.[10] We obtained this cell line from Dr. Young S. Kim of the Veterans Administration Hospital, San Francisco, who is maintaining it in his laboratory and has determined that it contains CEA on its surface and releases CEA into the medium.

Initially we evaluated subculture conditions that would allow SK-CO-1 cells to reach confluency on a 75-cm^2 monolayer in 6 to 7 days. SK-CO-1 cells were grown in Dulbecco's minimal essential medium with high glucose (4.5 g/ℓ), supplemented with 10% fetal calf serum (DMEM-HG/10), and incubated at 37° C in an atmosphere of 5% carbon dioxide-95% air. Confluent monolayers (75 cm^2) were treated with 0.25% trypsin-0.1% EDTA (trypsin-EDTA) to release the cells. The suspended cells were diluted 1 to 3, 1 to 4, and 1 to 5, were replated in 75-cm^2 flasks, and were allowed to adhere overnight. Spent culture medium was replaced with fresh DMEM-HG/10 3 days after plating, when the phenol red indicator in the medium had turned yellow, indicating that a considerable amount of lactic acid had been produced.

Cells diluted 1 to 3 were confluent in 4 days, cells diluted 1 to 4 were confluent in 5 days, and cells diluted 1 to 5 were confluent in 6 days. We chose a subculture dilution of 1 to 5 to study cell growth and are also using this dilution for routine maintenance of the cell line.

To study cell growth, we prepared fourteen 75-cm^2 flasks of SK-CO-1 cells at a subculture dilution of 1 to 5. This was equivalent to an initial cell inoculum of 4.2×10^6 per flask. At 24-hour intervals after subculturing, for a period of 7 days, the flasks were removed from the incubator, and the cells were released from the monolayer with trypsin-EDTA and counted in a hemocytometer. The cell counts from duplicate flasks were averaged. Spent culture medium in the ten flasks remaining on Day 3 was replaced with DMEM-HG/10 as described above.

The results of this experiment for the first 6 days of culture are illustrated in Figure 1. In this experiment, a loss of cells was observed 24 hours after subculturing. This occurs occasionally and is

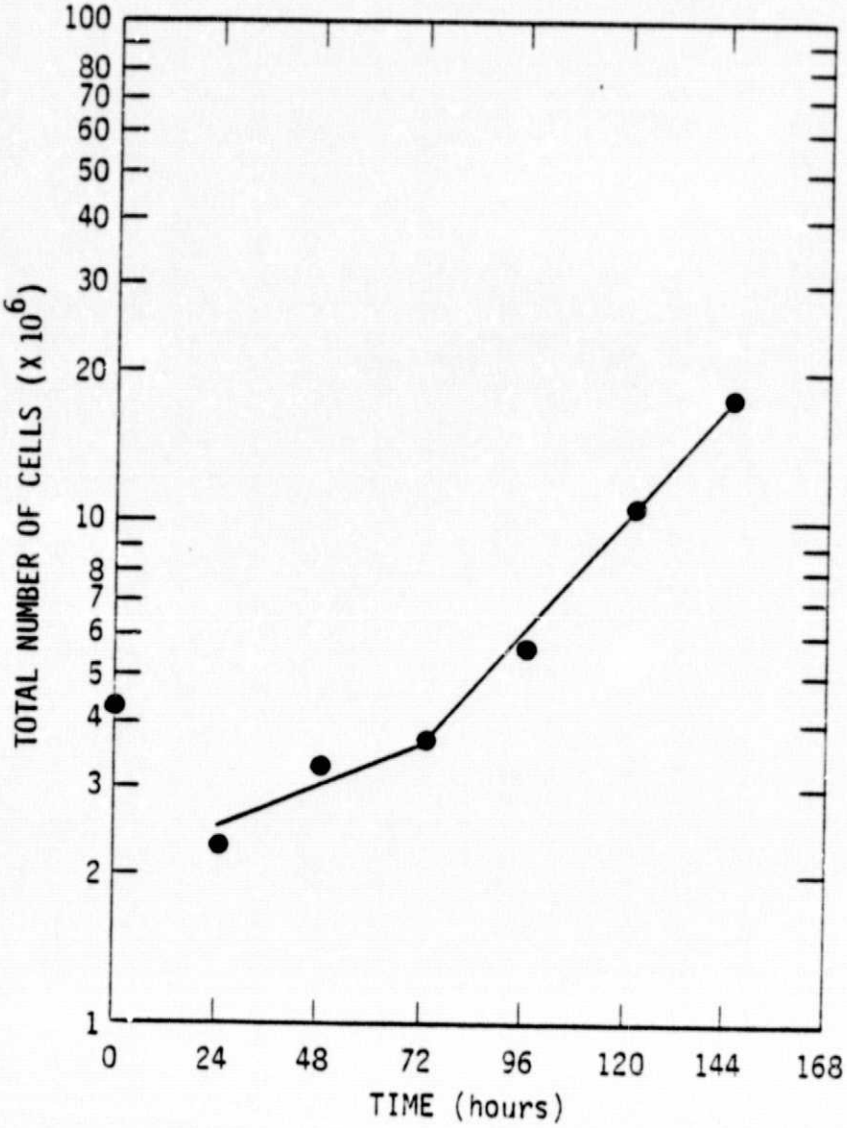

FIGURE 1 GROWTH OF SK-CO-1 CELLS IN MONOLAYER CULTURE

probably due to cell death resulting from damage by trypsin-EDTA that cannot be controlled experimentally. The doubling time calculated in this experiment was the same as that calculated in an experiment in which this initial cell loss did not occur (see Figure 2). Therefore, the initial cell loss does not effect doubling time calculations.

The plot illustrated in Figure 1 is a typical biphasic growth curve. A lag phase was observed for the first 3 days after subculture and logarithmic growth occurred for the next 3 days, at which time the cells reached confluency. The average number of cells per flask increased from 2.3×10^6 on Day 1 to 1.7×10^7 on Day 7, a 7.3-fold increase. The equivalent cell density was $3.1 \times 10^4/cm^2$ on Day 1 and $2.2 \times 10^5/cm^2$ on Day 7. The doubling time calculated from this plot was 88.8 hours during the lag phase and 33.2 hours during logarithmic growth.

To determine the amount of cell-bound CEA and the CEA released we grew SK-CO-1 cells in monolayer cultures as described above. Every 24 hours after subculturing, for 6 days, cultures were removed from the incubator, the medium was removed and stored at -80° C, and the monolayers were washed twice with phosphate-buffered (0.02 M, pH 7.2) saline (0.15 M). Then a minimal volume of phosphate-buffered saline was placed over the monolayer, and the cells were dislodged by scraping with a rubber policeman. The dislodged cell suspension was removed, and the flasks were washed with small amounts of phosphate-buffered saline and scraped with a rubber policeman to collect any remaining cells. The washings were combined with the initial dislodged cell suspension and stored at -80° C.

CEA analysis was performed by radioimmunoassay, using reagents purchased from Hoffman-La Roche, Inc., Nutley, New Jersey. The procedure used was developed by Drs. Dean Tsao and Young S. Kim (personal communication) of the VA Hospital in San Francisco, California, for measuring CEA on cultured colon carcinoma cells; it is a modification of the Hoffman-La Roche assay. By this procedure, the dislodged cell suspension is dispersed by sonication and diluted with a 5% solution of the detergent NP-40 to solubilize subcellular particles. The detergent-solubilized material is sonicated again and diluted with 0.01 M ammonium acetate (pH 6.5) so that the CEA content of a 0.1-ml aliquot is within the range of values detectable by the radioimmunoassay. Samples of the culture medium are diluted 1 to 20 with 0.01 M ammonium acetate (pH 6.5) to reduce the concentration of fetal calf serum glycoproteins to a level at which they will not interfere with the CEA assay. The radioimmunoassay for CEA is carried out essentially as described by Hoffman-La Roche, except that the amounts of each of the reagents used are adjusted so that quantities of CEA in the range 125 to 875 picograms can be detected.

The results of this experiment are illustrated in Figure 2. The total CEA content of SK-CO-1 cells 24 hours after subculturing was 5.6 μg; the content increased with time after subculturing, reaching a value of 28.5 μg on Day 6. When total cellular CEA values of cultures analyzed on Days 1 through 6 were expressed as CEA per 10^6 cells (Table 1), the values were

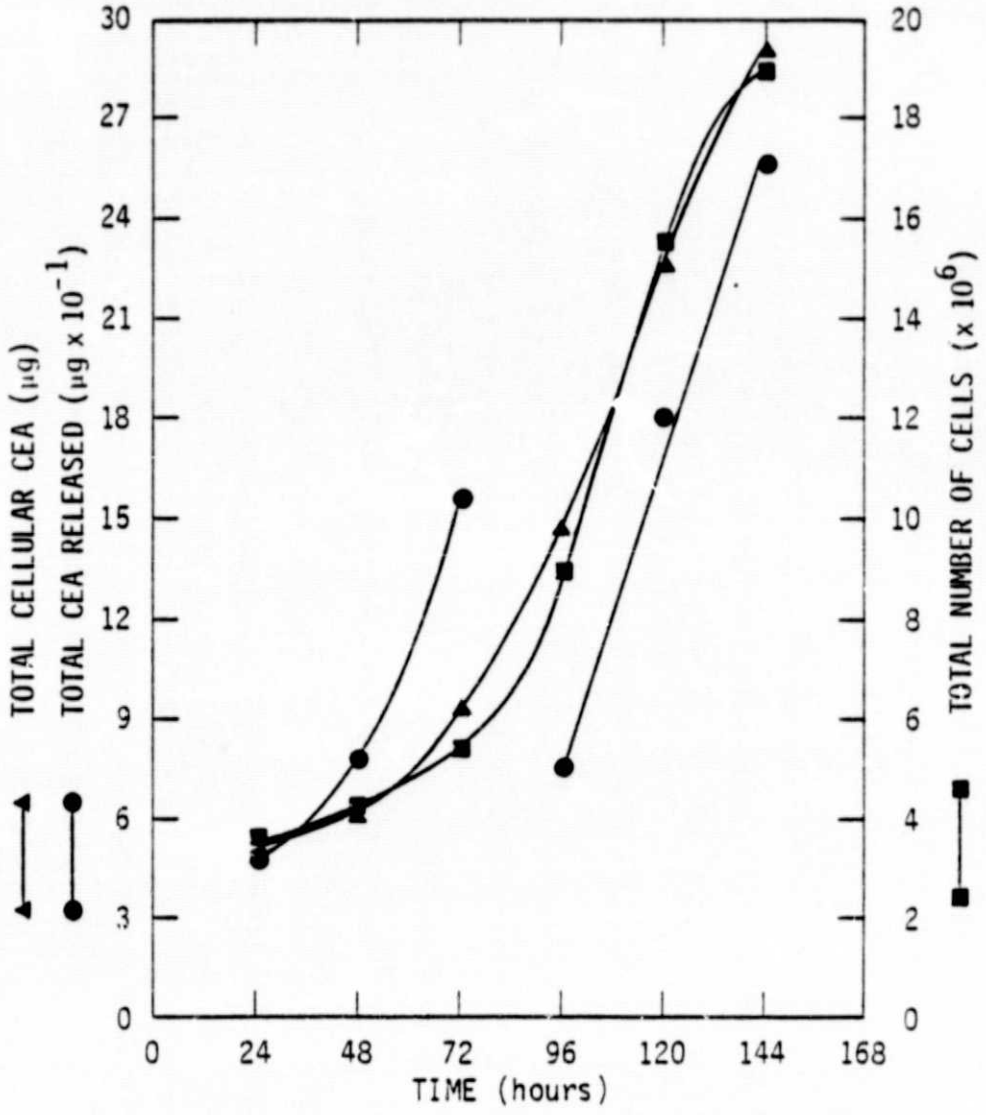

FIGURE 2 CEA PRODUCTION AND RELEASE AS A FUNCTION OF SK-CO-1 CELL GROWTH

similar. Therefore, the production of CEA by SK-CO-1 cells grown in monolayer cultures does not vary as a function of cell growth, and the

Table 1

CEA CONTENT PER UNIT NUMBER (10^6) OF SK-CO-1 CELLS GROWN IN MONOLAYER CULTURE

Day of Experiment	Total No. Cells ($\times 10^6$)	Total CEA Content (μg)	CEA/10^6 Cells (μg)
1	3.65	5.60	1.53
2	4.44	6.10	1.37
3	5.40	9.45	1.75
4	9.02	14.60	1.62
5	15.60	22.50	1.44
6	19.10	28.50	1.49

Average CEA content/10^6 cells - 1.53 ± 0.13 μg.

increase in total cellular CEA content with time is due to an increase in the number of cells. From the data obtained in this experiment, we calculated the average cellular CEA content to be 1.53 ± 0.13 $\mu g/10^6$ cells.

The total CEA released into the culture medium (20 ml) during the first 24 hours after subculturing was 0.56 μg (Figure 2). This increased to 0.82 μg on Day 2 and to 1.6 μg on Day 3 when the medium of all the remaining cultures was changed. Within 24 hours after changing the medium (Day 2), 0.82 μg of CEA was released, and the amount of released CEA increased linearly to 1.8 μg on Day 5 and 2.6 μg on Day 6. Therefore, the increase in CEA content of the medium is proportional to the increase in the number of cells.

Task 2 - Evaluation of CEA Production and Release by SK-CO-1 Cells Grown on Glass Beads

In space, glass beads will remain suspended in liquid medium. Suspension of these beads in unit gravity requires vigorous stirring, which creates a shear force that damages cultured cells. Therefore, we investigated the growth of SK-CO-1 cells on single layers of glass beads placed in plastic, bacterial petri dishes. These dishes are manufactured from a plastic material to which cells will not adhere; hence, the cells will adhere to and grow only on the glass beads. The layer of glass beads approximates a cross-section through a large vessel containing many layers of glass beads in space.

We determined that the quantity of glass beads with an average diameter of 200 μ needed to cover the bottom of a 21-cm^2 bacterial petri dish was 0.35 g. Aliquots of this weight of glass beads were autoclaved and placed in the dishes. Then four milliliter suspensions of SK-CO-1 cells in DMEM-HG/10, obtained from monolayer cultures by trypsinization, were added at densities of 5×10^5/ml, 2.5×10^5/ml, or 1×10^5/ml. The lids were placed on the dishes, which were then incubated at 37° C in an atmosphere of 5% carbon dioxide-95% air. All cultures were examined daily under a microscope and the medium was changed when necessary.

At all the cell densities used, cells adhered to the majority of the glass beads after 24 hours. Furthermore, by that time, the cells on the beads had flattened and had proceeded to divide. However, several differences were noted between the cultures. Beads in cultures initiated at a cell density of 5×10^5/ml were completely covered with cells in 4 days and extensive bridging of most beads by the cells occurred, creating a sheet of glass beads that was difficult to disperse by gentle agitation; very few individual cell-coated beads were observed. Beads in cultures initiated at a cell density of 2.5×10^5/ml did not show this extensive bridging but formed small clusters, with many free cell-coated beads. The cells completely coated these beads in 7 days. Similarly, cultures initiated at cell densities of 1.5×10^5/ml showed very little bridging of beads by cells, and the beads in these cultures were also largely covered by cells 7 days after initiation.

For cell growth experiments, cultures were initiated with a total of 4×10^5, 1×10^6, or 2×10^6 SK-CO-1 cells. Fourteen dishes were used for each of the initial seedings. Every 24 hours after the initial seeding, two dishes were withdrawn from the incubator. The medium was removed and the glass beads were suspended in 0.1 M citric acid containing 0.1% crystal violet and then incubated for 1 hour at 37° C. This reagent disrupts the cells and stains the nuclei purple. Therefore, the number of cells that grew on the glass beads could be determined by counting the nuclei in a hemocytometer.

Figure 3 illustrates the growth curves obtained. Cultures initiated with 4×10^5 cells were stationary for 24 hours and then began exponential growth, which lasted for 4 days; the cells then entered the lag phase, which lasted an additional 2 days. The total number of cells increased to 2.8×10^6 on Day 7, a 7-fold increase. Cultures initiated with 1×10^6 cells had a lag period and logarithmic growth period similar to those of cultures initiated at 4×10^5 cells. However, these cultures were stationary after 5 days and the number of cells increased to 4×10^6 on Day 5, a 4-fold increase.

In cultures initiated at 2×10^6 cells, an initial loss occurred during the first 48 hours after incubation; the number of cells decreased from 2×10^6 to 1.5×10^6. Then exponential growth began and continued for 3 days, at which time the cultures became stationary. After the intial loss, the number of cells increased to 7×10^6, a 5-fold increase. However, the final number of cells represents only a 3.5-fold increase over the number originally seeded.

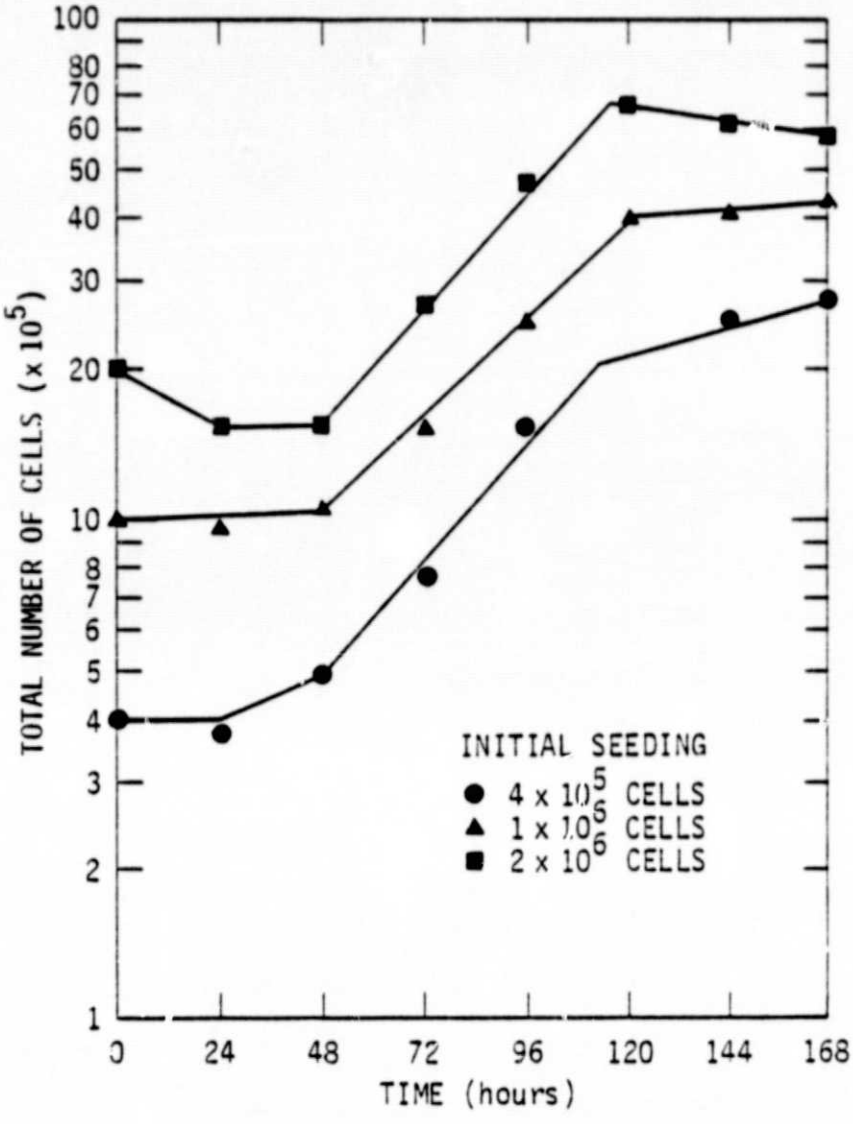

FIGURE 3 GROWTH OF SK-CO-1 CELLS ON GLASS BEADS

All growth curves were essentially parallel during the exponential growth phase, indicating that the cell doubling time was similar for all three conditions of cell growth. We calculated an average doubling time for the three experiments of 33.6 hours; this agrees well with that obtained for SK-CO-1 cells grown in monolayer culture.

The weight of glass beads used in these experiments (0.35 g) represents an average of 3.42×10^4 beads, as determined by counting an aliquot under the microscope. If the average diameter of the beads is 200 μ, the surface area per bead is 0.126 mm^2. Therefore, 0.35 g of beads has a surface area of 43 cm^2. We used this value to calculate cell density per unit area for each of the three conditions of growth and for comparison with cells grown in monolayer culture. The cell density of cultures initiated with 4×10^5 cells increased from 1×10^4 cells/cm^2 at initiation to 6×10^4 cells/cm^2 on Day 7. Cultures initiated with 1×10^6 cells increased from 2×10^4 cells/cm^2 at initiation to 1×10^5 cells/cm^2 on Day 6. Finally, cultures initiated at 2×10^6 cells decreased from 5×10^4 cells/cm^2 at initiation to 3×10^4 cells/cm^2 on Day 2 and then increased to 1.6×10^5 cells/cm^2 on Day 5.

The density of SK-CO-1 cells grown in 75-cm^2 flasks in monolayer culture increased from 3.1×10^4 cells/cm^2 to a maximum of 2.2×10^5 cells/cm^2. The maximum density achieved on beads in these experiments is 27 to 73% of that achieved in a monolayer culture. These differences may be due to the fact that the initial attachment of cells to beads in stationary cultures is uneven. (A microscopic examination of cultures during these experiments revealed that cells of many beads reached confluency during the early phases of growth, whereas cells on other beads were not confluent even after growing 7 days.) Overcoming this problem of uneven distribution in stationary culture should be possible in suspension culture, as should achievement of cell densities comparable to or greater than those obtained in monolayer culture. However, even under the conditions we used, the surface area per unit volume of medium that can be achieved in bead cultures more than compensates for the lower cell density.

For analysis of cellular CEA content and CEA released into the medium, we placed a 0.92-g aliquot of sterile glass beads into twenty-eight 60-cm^2 bacterial petri dishes. This quantity of beads is equivalent to 113.3 cm^2. Then, 2.6×10^6 SK-CO-1 cells suspended in 10.5 ml of medium were added to the dishes. The initial number of cells and the number of beads used in this experiment were larger than those used in the previous experiment to ensure that there would be enough CEA to measure accurately.

The dishes were incubated at 37° C in a humidified atmosphere containing 5% carbon dioxide-95% air. Each day for 7 days, four petri dishes were removed from the incubator. Cell counts were performed on two of the petri dishes by removing the medium, suspending the bead-bound cells in 0.1 M citric acid containing 0.1% crystal violet, and counting the stained nuclei. The other two petri dishes were used for analysis of cell-bound CEA and CEA released into the culture medium. The spent culture medium was replaced with fresh medium in all dishes remaining 3 days after initiation of the cultures.

For CEA analyses, the medium was removed from duplicate cultures and stored at -80° C. Then the glass beads containing SK-CO-1 cells were suspended in phosphate-buffered (0.02 M, pH 7.2) saline (0.15 M) containing 5% NP-40 and disrupted by sonication. A microscopic examination of the glass beads after sonication did not reveal any intact cells. The cell lysate was stored at -80° C until needed. Cell-bound CEA and CEA released into the medium were measured by a radioimmunoassay with reagents purchased from Hoffman-La Roche, Inc., Nutley, New Jersey. The assay was modified as described above.

Table 2 illustrates the results of this experiment. The total CEA content of SK-CO-1 cells 24 hours after culturing was 2.6 μg; the content increased with time, reaching a maximum of 9.4 μg on Day 6. When total cellular CEA values analyzed on Days 1 through 6 were expressed as CEA per 10^6 cells, the values were similar. Therefore, the production of CEA by SK-CO-1 cells grown on glass beads does not vary as a function of cell growth and the increase in total cellular CEA with time is due to an increase in the number of cells. The same observation was made when cellular CEA content of SK-CO-1 cells grown in monolayer culture was analyzed (Table 1).

From the data obtained in this experiment, we calculated that the average cellular CEA content was 1.38 ± 0.10 μg/10^6 cells. The value obtained for monolayer cultures of SK-CO-1 cells was 1.53 ± 0.13 μg/10^6 cells. Therefore, growing SK-CO-1 cells on glass beads does not have any gross effects on their expression of membrane-bound CEA.

The total CEA released into the culture medium during the first 24 hours of culture was 0.64 μg; this increased to 1.41 μg on Day 3 when the culture medium was changed. Within 24 hours after the medium was changed (Day 4), 0.87 μg of CEA was released; the amount increased thereafter, reaching a maximum value of 2.01 μg on Day 7. The increase in CEA released by SK-CO-1 cells grown in monolayer culture reached a maximum of 2.6 μg on Day 6. However, this amount of CEA was released by a culture that contained 2 x 10^7 cells at maximum growth, whereas CEA released by cells grown on glass beads was from a culture that contained 7.6 x 10^6 cells at maximum growth. If we compare the amount of CEA released by the two types of cultures on this basis, we can conclude that SK-CO-1 cells grown on glass beads release approximately 2.5 times more CEA than do cells grown in monolayer cultures. This finding is reproducible.

In this task, we established that the doubling time of SK-CO-1 cells grown on glass beads in stationary culture and their cellular CEA content are similar to those values obtained when the cells are grown in monolayer cultures. However, a larger amount of CEA is released into the medium when cells are grown on glass beads. Although the increase in cell density is slightly less in glass bead cultures than in monolayer cultures, we believe that this is due to the uneven distribution of the cells on the glass beads in stationary culture and that this problem could be overcome by using glass beads in suspension culture. Because

Table 2

CELL-BOUND CEA AND CEA RELEASED FROM SK-CO-1 CELLS GROWN ON GLASS BEADS

Day of Experiment	Total No. Cells ($\times 10^6$)	Total Cell-Bound CEA (µg)	Cell-Bound CEA/10^6 Cells (µg)	Released CEA (µg)
1	1.75	2.6	1.44	0.64
2	2.00	3.0	1.50	1.12
3	2.77	4.8	1.48	1.41
4	4.81	6.6	1.37	0.87
5	5.49	7.2	1.29	1.54
6	7.61	9.4	1.24	2.01

Average CEA content/10^6 cells - 1.38 ± 0.10 µg.

spherical microcarriers provide a means of obtaining a larger surface area per unit volume of culture medium than does any type of monolayer culture, we believe that our results demonstrate their usefulness for growing large numbers of cultured cells to produce materials that are of value as pharmaceuticals.

Task 3 - Evaluation of Other Microcarriers for Growing SK-CO-1 Cells and Determination of the Minimum Amount of Culture Medium Needed for Cell Growth

Originally we proposed to define requirements for growing SK-CO-1 cells on glass beads that would have included redox potential of the medium at different stages of cell growth, the sensitivity of cells to pH changes, the effect of specific metabolites on CEA production, and the effect of repeated medium changes on CEA production. In February, 1978, the principal investigator visited Dr. Dennis Morrison at the Johnson Space Center and discussed the results of Tasks 1 and 2. They discussed the problems of growing SK-CO-1 cells on glass beads in unit gravity and agreed on the importance of developing a microcarrier bead suspension culture on earth before experimenting in space. However, this would require using microcarriers that can be suspended by gentle stirring. They agreed that finding such a microcarrier would take precedence over experiments originally proposed for Task 3. They also agreed that the minimum amount of culture medium needed to grow the cells should be determined because the total amount of medium used has an impact on the economics of cell production.

In a recent report, Levine et al.[11] described a low-ion-exchange-capacity DEAE-Sephadex microcarrier for growing secondary chicken embryo fibroblasts and normal diploid human fibroblasts in suspension. They estimated that 1 gram of dry beads contains 6100 cm^2 of surface area and is equivalent to 110 100-mm petri dishes or 12 standard roller bottles. We prepared these microcarriers with diethylaminoethyl chloride and Sephadex G-50 according to their procedure and evaluated them for growing SK-CO-1 cells in suspension, using the cell-to-bead ratio they recommended.

SK-CO-1 cells stirred in suspension with these microcarriers remained viable, were metabolically active, and produced lactic acid. However, microscopic examination of samples of these cultures after 7 days of incubation revealed large clumps of cells that were not adhering to the suspended microcarriers. Most beads had only two or three adherent cells and these had not flattened onto the surface and were not proliferating.

We investigated the growth of SK-CO-1 cells on these microcarriers in stationary culture to determine whether the failure of these cells to grow on these beads was due to stirring or to an intrinsic property of the beads. In these experiments we also used DEAE-Sephadex A-50 (purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey) which has a

high-ion-exchange capacity (3.5 meq/g). Before using these beads for tissue culture, they were washed extensively with phosphate-buffered saline, autoclaved, and presoaked in fetal calf serum to attenuate any intrinsic toxic properties.

Low- or high-ion-exchange-capacity DEAE-Sephadex beads were placed in 21-cm^2 bacterial petri dishes. The amount of beads used was sufficient to cover the bottom surface of the dish. Then 4 ml of a suspension of SK-CO-1 cells at a density of 5×10^5/ml of culture medium was added. The lids were placed on the dishes, which were then incubated at 37° C in an atmosphere of 5% carbon dioxide-95% air. All cultures were examined daily under a microscope, and the medium was changed when necessary.

Large numbers of SK-CO-1 cells adhered to the high-ion-exchange-capacity beads, causing the beads to clump. Fewer cells adhered to the low-ion-exchange-capacity beads, which did not clump. In both cultures, the cells were metabolically active and produced lactic acid. However, they exhibited a round morphology for approximately 2 weeks; then they began to flatten onto the surface of the beads and proliferate very slowly. This contrasts with SK-CO-1 cells grown in monolayer cultures or on glass beads where cells adhere to the surface of the culture vessel or bead, flatten within 24 hours, and begin to proliferate actively. Therefore, the failure of SK-CO-1 cells to grow in DEAE-Sephadex microcarriers is due to an intrinsic property of the bead, not to stirring.

We also attempted to grow SK-CO-1 cells in suspension without microcarriers, on Bio-Gel P-30 (polyacrylamide) beads, and on Biosilon microcarriers manufactured by A/S Nunc, Copenhagen, Denmark. Cells grown in suspension were seeded at 5×10^5/ml in a spinner flask and stirred slowly using an ABS Polystirrer (Associated Biomedic Systems, Inc., Buffalo, New York). Within 24 hours after seeding multicellular clumps of viable cells were noted. Cells in these clumps remained viable for one week and then began to die. By the end of the second week of culture, all cells were dead. The SK-CO-1 suspension culture was sampled daily and the number of cells was determined. We found that it was necessary to treat the cells with trypsin-EDTA to break up the clumps so we could obtain an accurate cell count. The number of cells remained constant during the first week of culture and declined during the second week as cells died. Thus, SK-CO-1 cells do not grow in suspension without microcarriers.

We attempted to grow SK-CO-1 cells on Bio-Gel P-30 beads because Dr. James Allison of the University of Texas System Cancer Center, Smithville, Texas, advised us that tumor cells will grow in dishes cast of polyacrylamide. Bio-Gel P-30 beads were hydrated in phosphate-buffered saline and sterilized by autoclaving. Sufficient beads to cover the bottom were placed in bacterial petri dishes and suspensions of SK-CO-1 cells were added to the dishes. Repeated observations indicated that the cells did not adhere to the beads and grow. Therefore, polyacrylamide beads are not suitable as microcarriers for growing SK-CO-1 cells.

Biosilon microcarriers are negatively charged solid plastic spheres that range in size from 160-300 μ. They are specifically fabricated for growing tissue culture cells from a material similar to that used for manufacturing tissue culture flasks. We attempted to grow SK-CO-1 cells on these microcarriers in bacterial petri dishes using the conditions recommended by the manufacturer. Cells attached to the microcarrier surface, flattened within 24 hours, and remained viable for two weeks. However, they did not replicate indicating that Biosilon microcarriers do not support the growth of SK-CC-1 cells.

We discussed our lack of success with synthetic microcarriers with Dr. William McLimans of the Roswell Park Memorial Institute, Buffalo, New York. He said that his experience has indicated that many of these do not support the growth of cells and there is no microcarrier that will support the growth of all cultured cells. However, he has been experimenting with collagen particles as microcarriers for growing cells and has found that they are highly effective substrates. If procedures could be developed to make collagen spheres, these could be useful as microcarriers for large-scale growth in suspension of adherent cultured cells.

Dr. Dennis Morrison requested that we determine the minimum amount of culture medium needed to grow SK-CO-1 cells in stationary glass bead cultures and in monolayer cultures before evaluating their growth on collagen. Stationary glass bead cultures are grown in bacterial petri dishes that are made of a plastic material that has a high surface tension. Small amounts of liquid placed in these dishes form blebs that do not cover all the beads. Therefore, the least amount of medium needed for growth of these cultures could not be determined.

Tissue culture flasks for monolayer cultures are made of a material that is more wettable than the material used for bacterial petri dishes. The minimum amount of liquid that covers the surface of a 75-cm^2 tissue culture flask is 5 ml. Using this volume of medium we evaluated the growth of SK-CO-1 cells in monolayer cultures by methods described in Task 1. The cell doubling time was 35.2 hours, a value similar to that obtained when cultures were grown in 20 ml of medium. However, the phenol red in the medium turned yellow sooner in these cultures than in those that were grown previously in 20 ml of medium. Therefore, the culture medium had to be changed every 2 days. In the seven day period needed for these cultures to reach confluency four aliquots of medium of 5 ml each or a total of 20 ml of medium was used, whereas previously a total volume of 40 ml of medium was chosen arbitrarily. This experiment demonstrates that smaller volumes of culture medium than those used previously do not adversely effect the growth of SK-CO-1 cells in monolayer cultures. It also establishes a lower limit for the volume of medium required to grow SK-CO-1 cells as monolayers.

Task 4 - Growth of SK-CO-1 Cells on Collagen Monolayers and CEA Production

Before attempting to fabricate microspheres out of collagen we studied the growth of SK-CO-1 cells and their production of CEA on collagen monolayers. Collagen was extracted from rat tail tendons with 0.1% acetic acid by the procedure of Hauschka and Konigsberg[12] and collagen gels were prepared as described by these authors. Briefly, into 79-cm^2 bacterial petri dishes was placed 0.05 ml of rat tail collagen in 0.1% acetic acid (0.7 mg protein/ml). Then 0.01 ml of a 6% sodium chloride solution was added and the two solutions were mixed and spread over the bottom of the dishes with a glass stirring rod. Gelation was accomplished by placing the dishes in a humidified desiccator overnight. The dishes were washed twice with saline and once with medium before use.

The consistency of gels produced by this procedure was poor; they did not stick to the surface of the petri dishes and tended to float when washed or when culture medium was added. This problem could be overcome if gelation was induced by placing the dishes overnight in a desiccator containing a paper towel soaked in concentrated ammonium hydroxide and washing as described above.

Ten-ml suspensions of SK-CO-1 cells (5×10^5/ml) in DMEM-HG/10 were placed on collagen monolayers and incubated at 37° C in a humidifed atmosphere of 5% carbon dioxide-95% air. Cultures were examined daily for 15 days for morphology and for evidence of cell growth. The cells attached to the monolayers within the first 5 hours of culture and replicated. However, they did not completely flatten. Instead they exhibited a rounded morphology that persisted for the entire period of culture. In addition, they did not grow to confluency; after 15 days of culture there were areas of the collagen monolayer that were devoid of cells or that contained very few cells. Increasing the concentration of collagen in the gels did not overcome this suggesting that the initial attachment of cells to the monolayer was uneven or that gelation was not uniform. Because initial observations of the cultures indicated that cell attachment was uniform we concluded that gelation was uneven. Therefore, we devised an alternative procedure for producing collagen gels.

Acetic acid extracts of rat tails were prepared by the procedure of Hauschka and Konigsberg[12], lyophilized, and stored frozen. Then the lyophilized collagen was suspended in ice-cold sterile 0.075 M citrate buffer, pH 4.4, and dissolved by stirring slowly at 4° C for 48 hours. This solution was placed in an ice bath and a sufficient amount of cold, sterile, 0.5 M sodium carbonate was added to bring the pH to 7.2. Three-ml aliquots of the neutralized collagen solution were transferred to 79-cm^2 bacterial petri dishes and spread over their surface; incubating the plates at 37° C for 4 to 5 hours caused gelation.

SK-CO-1 cells grew to confluency on 1% collagen gels prepared by this procedure so it could be used to prepare gels to analyze their growth and production of CEA on this substrate. However, the cells still exhibited

a rounded morphology suggesting that their morphology is governed by the substrate on which they grow.

To study cell growth, we prepared 1% collagen gels in 79-cm^2 bacterial petri dishes and inoculated them with 2.1 x 10^6 cells. At 24-hour intervals, for a period of 14 days, duplicate dishes were removed from the incubator and the cells were released from the monolayer as a sheet by treating it with collagenase (4.5 mg in 3 ml of DMEM-HG/10) for 10 minutes at 37° C. Then a single cell suspension was prepared from the sheet of cells by treating it with trypsin-EDTA and the suspension was counted in a hemocytometer. Values from duplicate dishes were averaged.

Figure 4 illustrates the results of this experiment. Also included for comparison is the growth curve from Figure 1 of SK-CO-1 cells grown as monolayers in plastic flasks. Cells grown on collagen monolayers did not exhibit a biphasic growth curve. Their doubling time was 62 hours which is 1.9 times longer than that of SK-CO-1 cells during logarithmic growth in plastic flasks. Moreover, cells grown on collagen monolayers took 12 days to reach confluency whereas cells grown as monolayers in plastic flasks reached confluency in 6 days.

The average number of cells per 79-cm^2 collagen monolayer increased from 2.6 x 10^6 on Day 1 to 5.4 x 10^7 on Day 12, a 21-fold increase. The equivalent cell density was 3.1 x $10^4/cm^2$ on Day 1 and 6.9 x $10^5/cm^2$ on Day 12. Thus, the increase in the number of SK-CO-1 cells grown on collagen was 3-fold greater than that of SK-CO-1 cells grown on plastic.

We also analyzed cellular CEA and CEA released into the medium. SK-CO-1 cultures were established on collagen monolayers as described above. Every 24 hours four dishes were removed from the incubator; two were used for counting cells and two for CEA analysis. Medium was removed from the latter cultures and stored at -80° C. Then the monolayers were treated with collagenase. The sheet of cells was collected by centrifugation, washed twice with phosphate buffered (0.02 M, pH 7.2) saline (0.15 M), and suspended in 2 ml of the same buffer. Suspensions were stored at -80° C.

CEA analysis was performed as described in Task 1 except that the medium was not diluted; duplicate values for each determination were averaged.

Table 4 illustrates the results of this experiment. The total CEA content of SK-CO-1 cells 24 hours after subculturing was 1.24 μg; the content increased with time after subculturing, reaching a value of 38.4 μg on Day 12. When total cellular CEA values analyzed on Days 1 through 12 were expressed as CEA per 10^6 cells, the values were similar and the average value was 0.74 ± 0.10 $\mu g/10^6$ cells. This is 48% of the cellular CEA content of SK-CO-1 cells grown in plastic tissue culture flasks.

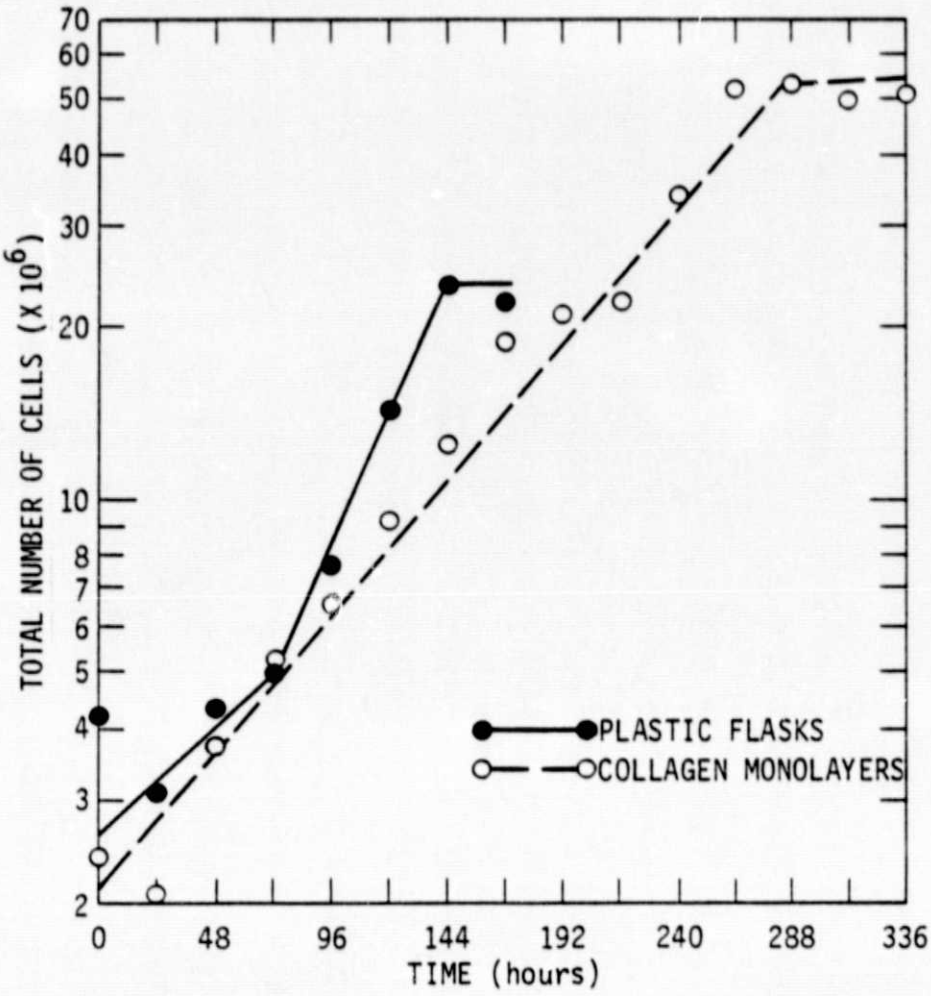

FIGURE 4 COMPARISON OF SK-CO-1 CELLS GROWN IN PLASTIC FLASKS AND COLLAGEN MONOLAYERS

Table 4

CEA CONTENT PER UNIT NUMBER (10^6) OF SK-CO-1 CELLS GROWN IN MONOLAYER CULTURE

Day of Experiment	Total No. of Cells ($\times 10^6$)	Total CEA Content (μg)	CEA/10^6 Cells (μg)
1	1.57	1.24	0.79
2	2.21	1.57	0.71
3	2.94	2.35	0.80
4	5.00	2.85	0.57*
5	4.76	4.24	0.89
6	7.86	5.50	0.70
7	8.36	5.85	0.70*
8	16.40	11.00	0.67
9	15.07	10.70	0.71*
10	27.00	17.55	0.65
11	37.82	29.50	0.78*
12	43.14	38.40	0.89

*Medium changed.

Average CEA/10^6 cells = 0.74 ± 0.10 μg.

CEA was not detected in the culture medium until 3 days after initiation. The amount of CEA detected on Days 3 through 10 was constant and the average value was 0.40 ± 0.23 μg per 10 ml of culture medium. This value increased to 1.25 μg on Days 11 and 12. Thus, when SK-CO-1 cells are grown on collagen gels they release significantly less CEA than when grown in plastic tissue culture flasks.

We attempted to make collagen microspheres by mixing CM-Sephadex A-50 beads with a 0.5% collagen at pH 4.4, isolating the beads and washing them with phosphate buffered saline. This procedure resulted in large clumps of beads that could not be dispersed without damaging their coat of collagen. (After dispersion by mechanical procedures large pieces of collagen could be seen floating free in the medium used to suspend the beads.) Time did not permit exploring other procedures for fabricating collagen microspheres.

CONCLUSIONS AND RECOMMENDATIONS

In this project we compared the rate of growth and production of CEA by SK-CO-1 cells grown in plastic flasks and on collagen monolayers. We also studied cell growth and CEA production of SK-CO-1 cells on glass beads, polyacrylamide beads, low-ion-exchange capacity DEAE-Sephadex beads, and Biosilon beads. The latter two materials have been specifically developed for use as microcarriers to grow adherent mammalian cells in suspension culture.

Profound differences in cell growth rate, cell morphology, cellular CEA content, and CEA released into the culture medium were observed when SK-CO-1 cells grown in plastic flasks were compared to those grown on collagen monolayers. Cells grown on collagen had a longer doubling time, appeared rounder, and contained less CEA than cells grown on plastic. In addition, they released less CEA into the culture medium. Thus, the cell substrate plays a role in determining the growth rate and the production of a specific macromolecule.

SK-CO-1 cells did not grow on any of the synthetic microcarriers for cell culture that are currently available. However, they did grow on glass beads with a growth rate and CEA content similar to that of cells grown in plastic tissue culture flasks. The surface of glass beads available per unit volume of medium is greater than that of a monolayer so cell-bound CEA can be obtained more economically by this method than from cells grown as monolayers. This economic advantage is amplified further because SK-CO-1 cells grown on glass beads release more CEA into the medium than SK-CO-1 cells grown on monolayers. Thus, even at 1 x g glass beads are definitely more advantageous for growing SK-CO-1 cells for CEA production than monolayer cultures.

Because glass beads can only be suspended at 1 x g by vigorous stirring and this is detrimental to the growth of mammalian cells we could not evaluate the growth of SK-CO-1 cells on microcarriers in suspension. This evaluation will be necessary before microcarrier cultures can be considered for space flight so limiting conditions for using this technology on earth can be defined and the advantage of growing cells at 0 x g can be determined. However, if SK-CO-1 cells do not grow on microcarriers suspendable at 1 x g and if glass beads are the only microcarriers that readily support their growth suspension culture experiments must be performed at 0 x g.

Collagen is a suitable substrate for growing SK-CO-1 cells and it is theoretically possible to fabricate microcarriers out of this material. However, SK-CO-1 cells grow more slowly on collagen monolayer cultures and produce less CEA than when grown in plastic tissue culture

flasks. If they exhibit the same growth rate and CEA production on collagen microspheres, these may not be optimum for growing SK-CO-1 cells in suspension and other substrates for growing them that can be fabricated into microspheres will have to be investigated.

Additional ground-based experiments are needed before cell culture in space can be considered. In addition to evaluating other substrates for growing cells the growth of SK-CO-1 cells and their production of CEA on microcarriers in both stationary and suspension culture must be studied. The effect of the rate of stirring, microcarrier density, and frequency of medium changes on cell growth and CEA production must also be evaluated. Nutritional requirements should also be investigated including different growth media, different concentrations of fetal calf serum, and the effect of adding specific nutrients. Investigation of the optimum carbon dioxide and oxygen tensions and the optimum redox potential for cell growth and CEA production will also be important.

A study of these parameters will identify the best conditions for growth of SK-CO-1 cells on earth and for production of CEA. It will also indicate any limitations inherent in this process that can be overcome in space.

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