Three-dimensional gel-supported native-state histoculture for evaluation of tumor-specific pharmacological activity: principles, practice and possibilities

A three-dimensional sponge-matrix-supported histoculture system is described that allows tissues to be cultured with the preservation of native-state three-dimensional tissue architecture and function. Over 25 types of human tumor types have been successfully cultured in this system with the maintenance of proliferative capacities, tumor architecture and function including tumor-stromal interactions. A number of normal tissues can also be successfully cultured in the system with the maintenance of tissue architecture and proliferative capacity. A drug-response assay has been developed with this system that allows over 80% of specimens from all tumor types to be evaluated. Accurate in vitro/in vivo drug-response correlations have been obtained using the native-state system. The system offers the possibility of low-cost predictive drug-response determinations for all cancer patients undergoing surgery or biopsy, as well as assays of other clinically-related parameters. The native-state system's ability to culture both tumor as well as normal tissue and assess their drug-response spectra enables the evaluation of the tumor-specificity of investigational anticancer agents.

Key words: In vivo-like growth — Predictive drug-response assay — New drug evaluation

Preface

Historical background of in vitro sensitivity-testing in cancer

There have been many attempts to design in vitro systems to culture tumor cells and tissues in order to design individual in vitro drug-response assays. A clonogenic assay was developed starting with the studies of Puck and Marcus [1], and leading to the studies in human myeloma of Hamburger and Salmon [2] as well as other tumor types. The clonogenic assay is based on the ability of disaggregated tumor cells to grow in agar or agar-like medium and form colonies. Drugs are termed effective if they inhibit formation of colonies. There have been many thousands of studies with the clonogenic assay, and there have been correlations between drug-response in vitro and clinical outcome. However, this assay has had a number of practical and theoretical problems including the low frequency of tumors yielding sufficient numbers of colonies to study drug response, artifactual problems such as clumping, which can be interpreted as colony formation, and the lack of tumor architecture and heterogeneous cell-cell interactions in the colonies which result [3, 4].

Other recent attempts have been made to develop drug-response assays for patients. These include modifications of clonogenic assays such as that
of Kern and Weisenthal [5] that measure \([1H]\) thymidine incorporation as an endpoint to determine drug response in cells incubated in an agar-based medium. The thymidine-incorporation clonogenic assay is found to be highly effective for detecting extreme drug resistance but not effective for detecting drug sensitivity. Other assays include those of Weisenthal where disaggregated cells in suspension are used to measure vital dye incorporation to determine drug effects on viability of cells [6]. The suspension-cell vital-dye assay seems more suited to hematological tumors than disaggregated solid tumors which will be in a very artificial environment.

The importance of three-dimensionality of cultured tissue is shown in many studies. For example, testing spheroids formed from cell lines in comparison with the same cell line in monolayer culture has indicated that spheroids are much more resistant to drugs than when the cells are in the monolayer state [7-12].

Rotman et al [13-15] have partially disaggregated tissues enzymatically into cellular clusters that they term microorgans which are claimed to be exclusively tumor cells. The microorgans are supported on cellulose fibers impregnated with collagen. Fluorescein-based vital-dye fluorescence is used as the endpoint for drug response in Rotman’s assay where only living cells will cleave the dyes to the fluorescent state. The assay seems promising in that the frequency of evaluation and clinical correlations for in vitro drug-response thus far are high.

Short-term assays such as that of Silvestrini and Sanfilippo where tumor fragments are incubated for three hours have also shown some clinical efficacy [16, 17]. The short-term assay uses incorporation of tritiated nucleic acid precursors as an endpoint to measure drug response. However, the viability of the tumors is limited in these assays.

The above-described attempts at developing in vitro sensitivity assays have indicated the need of a new generation of drug response assays that would be more representative of the in vivo state. Leighton [18] was instrumental in bringing tissue culture closer to the in vivo situation by developing the three-dimensional sponge matrix system. Leighton’s initial system was a cellulose-sponge matrix impregnated with a plasma clot and later modified [19] by using collagen-coated cellulose sponges. Leighton grew a number of tissues on cellulose-sponge matrices such as mouse mammary adenocarcinoma. The outgrowth of cells on the sponge matrix demonstrated multiple areas of organoid aggregations of cells which were very similar to the basic pattern of the tumor. Importantly the stromal elements were associated with some of the epithelial structures. Sponge elements provide physical support for the organized aggregations of cells such as the glandular formations of epithelial cells.

We have utilized collagen-containing sponges derived from pigskin to histoculture over 25 tumor types with the use of \([1H]\) thymidine incorporation as an endpoint to measure drug response. Histological autoradiography is used to measure \([1H]\) thymidine incorporation. This drug-response assay has been termed native-state, since tumor tissue architecture and functions are well preserved. The native-state system is a general one and should prove useful for all cancer patients. The assay has the following advantages [3, 20-24]:

— Cultures tissue in three dimensions, preserving native tissue architecture.
— Uses morphological end points.
— Detects proliferation by histological autoradiography and polarization microscopy yielding very high resolution data, the collection of which can be semi-automated by digital pixel analysis.
— Viability of tumor and non-tumor cells can be measured at high resolution by fluorescent-dye exclusion and inclusion detected in three-dimensions by confocal scanning-laser microscopy [25].
— Has a very high rate of evaluation for all tumor types—approximately, 80 % or better.
— Multiple agents can be tested sequentially.
— Uses relatively small amounts of tissue — as small as 100 mg.
— Has very low frequency of false negatives for drugs tested except for
References


mitomycin-C and low frequency of false positives for drugs tested except for 5-fluorouracil when using [3H]thymidine as an endpoint [26].

— Normal as well as tumor tissue can be cultured in parallel to assess the tumor-specificity of anti-neoplastic agents.

— Cytotoxic as well as biological drugs and activated immune cells can be assayed.

— Depending on end point chosen, data can be obtained in 3-14 days.

For detailed recent reviews of the various in vitro sensitivity assays previously or currently studied or utilized for cancer, please see the articles by Hoffman and by Von Hoff [3, 4].

Principles

The importance of the proper substrate for cultured tissues for nutrition, growth and maintenance of tissue architecture: sponges and collagen

Leighton [18-19, 27-28] in the 1950's developed the sponge-matrix technology for three-dimensional histoculture. Leighton has pointed out the following important aspects of using a sponge-matrix as a substrate for cultured tissues:

1) The interlocking sponge trabeculae, especially if coated with collagen or composed of collagen may allow a spatial arrangement enabling cells to grow in all directions, i.e., three dimensions. The sponge trabeculae also produce a rigid labyrinth upon which cells can migrate.

2) This labyrinthine structure in the sponge can create a very large increase in surface area which may favor growth of epithelial cells.

3) Cells forming aggregates within the sponge interstices may produce local reductions in metabolic exchanges giving the cells a nutritional advantage via utilization of their excreted products.

4) Local diffusion gradients may develop within interstices among the cell aggregates forming morphological gradients and thereby may influence the formation of differentiated features of the cultured tissues.

5) The sponge trabeculae may act as wicks and thereby provide mechanical pathways for metabolic exchanges, in effect producing a capillary-like circulation within the sponge. Sponges available include cellulose, collagen-coated cellulose and collagen. Leighton [29] indicated that collagen-coated cellulose may be the most effective substrates for in vivo-like tissue growth.

Maintenance of tissue architecture and tumor-stromal interactions in vitro

Leighton [15] has pointed out that in the United States microbiology has been the predominant point of view in studies of mammalian cells in tissue culture. The microbiological paradigm is emphasized in the use of monolayer cultures or suspension cultures of cell lines derived from human or animals. These systems enable large numbers of cells to grow that are easily quantified and used for cell and molecular biological experiments. The leading proponents of the microbiological paradigm have been and are virologists. The major problem with glass- or plastic-surface-grown cells or suspension-grown cells is that the cell types generally do not form histiotypic structures. The use of sponge-matrix culture, on the other hand, allows the formation and/or maintenance of tissue architecture in vitro. The significant point of sponge-matrix culture is that the multiple cell types comprising tissues are maintained in an in vivo-like structural arrangement where they can proliferate. Solid substrates such as glass or plastic in contrast promote the growth of fibroblasts which usually predominate mixed cultures of connective tissue and epithelium. Sponge-matrix culture allows a balance to be struck between the various cell types of a particular tissue that resembles the in vivo situation. Therefore, the critical process of tumor-stromal interaction is maintained in vitro in sponge-matrix culture.

With regard to tumor-stromal interactions, there is the possibility of each type of cell strongly influencing the other. Santesson [30] observed mutual stimulation between tumor and stromal cells in vitro. Ludford and Barlow [31] have also observed stimulation of connective tissue cells by carcinoma cells. Therefore, a realistic model of tumor culture should include stromal and tumor cells since they probably influence the growth of each
Other. Also the invasive properties of the tumor are affected by the stromal cells which is reflected in sponge-matrix culture [28]. In addition, Leighton [28] has indicated that in sponge-matrix tissue culture, progressively-increased numbers of aggregates of tumor cells may be produced in the absence of stroma. Perhaps the sponge itself is serving as a stroma which would not occur in monolayer cultures of tissue on plastic or glass.

**Tumor heterogeneity: problems and pitfalls**

It is now agreed upon that individual tumors may consist of many types of cells, both tumor and normal, and that they may have considerably different properties including drug response [32-42]. Indeed, some of the cells within a tumor may go on to dominate the tumor and produce metastases. Kerbel [43] has termed this phenomenon clonal dominance. The cells which eventually dominate a tumor and have the greatest metastatic potential, indeed, may be the most rapidly-growing cells in vitro as well. More directly to the point of in vitro drug-response assays, Murray et al [40] as long ago as 1954 have noted not only does drugs have different effects in vitro on tumors of comparable architecture from different patients but that the variation of responses to specific drugs can be seen in different areas of the same tumor. Therefore, it is important that a tumor-culture system be able to culture the multiple cell types of a tumor in their native architecture.

**Endpoint choices: advantages and disadvantages**

The proper in vivo-like growth of tumors in vitro is necessary for the design of an optimal drug-response assay but is not sufficient. The parameters or endpoints to measure drug response must be well chosen. The measurement of thymidine incorporation, for example, into cell nuclei allows the determination of the effects of drugs on DNA synthesis. Histological autoradiography allows the measurement of a morphological endpoint in that the cell types observed incorporating "H"thymidine by grain formation in the autoradiograms can be simultaneously identified mor-
point for in vitro drug response. See Fig. 1 to compare fluorescent-dye-based viability measurements observed with confocal microscopy with [H]thymidine uptake measured by histological autoradiography.

Oguro [46] has demonstrated that drug response may possibly be accurately measured by intracellular morphological changes observed by time-lapse video microscopy after drug treatment.

Practice

Types of tumors cultured in the native-state histoculture system

Table 1 shows that 27 types of tumors have thus far been cultured in the native-state system. The frequency of successful culture is about 80% showing the generality of use of the system.

Types of normal tissues cultured in the native-state histoculture system

Importantly, as demonstrated earlier by Leighton [28] normal tissues can often be cultured well in the native-state system. See Table 2 for list of normal tissue types cultured. The comparison of in vitro drug response of tumors to that of normal tissue enables the evaluation of the tumor specificity of investigational anticancer agents.

Preservation of histological architecture in native-state histoculture

A key aspect of the native-state histoculture system is that there is very often high maintenance of histological architecture of tissue histocultured in vitro compared to the original tissue in vivo. A study was made by our group in collaboration with Kubota’s group at Keio University in Tokyo which indicated very strong histological correlation between human tumor tissue grown in vitro in the native-state sponge-matrix histoculture system and grown in vivo in nude mice [26]. Figures 2a and b compare a typical human stomach carcinoma grown in vivo and in vitro. Figures 2c and d compare a typical human colon carcinoma grown in vivo and in vitro. Figures 2e and f compare a typical human breast tumor grown in vivo and in vitro [26]. Note in each case that the main aspects of the tissue architecture in vivo including the glandular structures are preserved very well in vitro [26]. In the breast tumor, the undifferentiated state of the tumor in vivo is preserved in vitro.

Possibility of long-term culture

Slocum et al [47] have demonstrated that a number of human tumor types obtained at biopsy and initiated in the native-state histoculture sponge-matrix system can be serially passaged from one gel raft to another for periods exceeding one-and-one-half years with maintenance of at least some tissue architecture. The serially-passaged histocultures appear to take on some of the properties of established cell lines, but in this case in tissue form without in vivo passage. These serially-passagable histocultures have been termed histolines. These include sarcoma, colon carcinoma and melanoma thus far.

In vivo/in vitro drug-response correlations

In a series of human tumors cultured in native-state sponge-matrix histoculture as well as in nude mice, it has been demonstrated that there is a greater-than-80% accuracy in predicting drug resistance in vivo using suprapharmacologic doses of drugs in

<table>
<thead>
<tr>
<th>Table 1. Tumor types cultured in the native-state in vitro gel-supported histoculture system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal</td>
</tr>
<tr>
<td>Bladder</td>
</tr>
<tr>
<td>Brain</td>
</tr>
<tr>
<td>Breast</td>
</tr>
<tr>
<td>Cervix</td>
</tr>
<tr>
<td>Colon</td>
</tr>
<tr>
<td>Rectum</td>
</tr>
<tr>
<td>Endometrium</td>
</tr>
<tr>
<td>Esophagus</td>
</tr>
<tr>
<td>Gall bladder</td>
</tr>
<tr>
<td>Head and neck</td>
</tr>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>Kidney</td>
</tr>
<tr>
<td>Larynx</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2. Normal tissue types cultured in the native-state in vitro gel-supported histoculture system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
</tr>
<tr>
<td>Breast</td>
</tr>
<tr>
<td>Lung</td>
</tr>
<tr>
<td>Colon</td>
</tr>
</tbody>
</table>

References

Fig. 1 a-f. The comparison of the drug-response endpoint of [³H]thymidine uptake measured by histological autoradiography to the viability endpoint of dye inclusion and exclusion measured by fluorescence of BCECF-AM (green) in live cells to propidium iodide uptake (red) in dead cells observed with scanning-laser confocal microscopy.

Tumors were removed surgically and placed into culture medium (Eagle's minimal essential medium containing Earle's salts, glutamine, 10% fetal calf serum, nonessential amino acids, and gentamicin) by the pathologist and transported to the laboratory. The tumors were minced into 1- to 2-mm-diameter pieces and planted on previously hydrated collagen-containing gel matrices (Health Design Industries, Rochester, NY) within 48 h of removal from the patient. Six 1-mm³ pieces of tissue, each representing a different region of the tumor, were explanted atop one 1 × 2 cm gel to overcome sampling error due to tumor heterogeneity.

[³H]Tdr (4 μCi/ml; 1 Ci = 37 GBq) was added to label replicating cells for 3 days starting on day two of culture. The cultures were then washed with phosphate-buffered saline, placed in histology capsules, and fixed in 10% formalin overnight. The histocultures were consequently dehydrated and rinsed with paraffin. The gel was then placed in an embedding cassette, then embedded so that all 6 pieces of tumor were at the front of the paraffin block. The block was then sectioned using a microtome so that a continuous ribbon of 5-μm sections was made, each containing 6 representative pieces of tumor. Approximately 10-15 sections of the ribbon were placed on each slide for autoradiographic processing and staining. Two slides were made of each condition. The slides (continued on p. 195)
were deparaffinized and then coated with Kodak NTR-2 emulsion in a dark room and allowed to expose for 5 days at 4 °C before developing. After rinsing, the slides were stained with hematoxylin and eosin.

For fluorescent-dye labeling of live and dead cells, viable cells are selectively labeled with the dye BCECF-AM, which is activated to fluorescence by non-specific esterases present only in living cells. Non-viable cells, whose plasma membranes are leaky, are labeled with propidium iodide (PI), a dye which enters only cells with non-intact membranes. Since the emission spectra of these two dyes are different they can be used simultaneously on the same specimen. Both dyes are used at a concentration of 5 μM. The double-dye-treated cultures are analyzed by fluorescence and confocal microscopy within 30 min of staining.

For scanning laser confocal fluorescence microscopy a Bio-Rad MRC 600 was used mounted on a Nikon Optiphot using a 10X PlanApo objective.

a Histological autoradiography of histocultured human colon tumor. Control — no drug treatment. Green grains, seen with polarization microscopy, indicate cell nuclei which have taken up [H]thymidine. b Same as a, except histocultures were treated with 5-fluorouracil. c Confocal microphotograph of histocultured colon tumor. Control — no drug treatment. Green cells, BCECF-AM-stained live cells. Red cells, propidium iodine-stained dead cells. d Same as e except histocultures were treated with 5-fluorouracil. Data are taken from reference [55]. e Confocal microphotograph of histocultured colon tumor. Control — no drug treatment. Treated with propidium iodine only. Red cells are dead. f Same as e except histocultures were treated with 5-fluorouracil.
Fig. 2 a-f. Comparison of histology of human tumors grown in native-state three-dimensional sponge-gel-matrix culture with the same human tumors grown in nude mice (See Fig. 1 for details). Tumors were grown in nude mice as described by Kubota et al. [53] 
(continued on p. 197)
a Human stomach tumor H-111 grown in nude mouse. Hematoxylin and eosin, 1000X. b H-111 grown in native-state sponge-gel-matrix histoculture. Autoradiogram. Hematoxylin and eosin, 1000X. c Human colon cancer Co-3 grown in nude mouse. Hematoxylin and eosin, 1000X. d Co-3 grown in native-state sponge-gel-matrix histoculture. See Fig. 2b for details. Data are taken from reference [26]. e Human breast tumor MX-1 grown in nude mouse, 1000X. f MX-1 grown in native-state sponge-gel matrix histoculture. See Fig. 2b for details.
vitro [26]. In other words, tumors that are resistant in vitro to large doses of drugs are very frequently resistant in vivo. The false-negative frequency is very low for tumors resistant to suprapharmacological doses of drugs in vitro with the exception of mitomycin C [26]. In addition, using pharmacological doses of drugs in vitro in native-state histoculture, a 65% accuracy in predicting drug response and resistance in vivo for the same human tumors in nude mice has been obtained. These results have all been obtained with the autoradiographic measurement of incorporation of [3H]thymidine as an endpoint [26]. Current experiments are ongoing with the use of fluorescent dye inclusion and exclusion to determine if viability measurements can lower the false-positive frequency.

Correlation of stage and grade with in vitro growth capacity in the native-state histoculture system

We have compared the ability of cancer tissue to proliferate in the native-state histoculture system to the stage and histological grade of four major types of human carcinomas: breast, ovarian, colon and lung. Eighty percent of tumor explants could be evaluated even when sent from across the country. We have determined that the growth fraction index which is determined by the percent of cells incorporating [3H]thymidine as measured by histological autoradiography (see below) correlates with tumor stage and grade for breast and ovarian carcinomas. In colon carcinoma there is a trend toward high growth fraction indices in tumors of more advanced stage and grade. In non-small-cell lung carcinomas, growth fraction index, stage and grade do not correlate. These results suggest the applicability of the sponge-matrix supported three-dimensional native-state histoculture system for prognostic purposes in patients with breast and ovarian cancers and demonstrate the clinical relevance of native-state histoculture [23].

The distinction of the growth potential of small-cell vs. non-small-cell lung tumors in native-state histoculture

Murray and Stout [39] were able to distinguish Ewing sarcoma, reticulum-cell sarcoma and neuroblastoma by the use of culture methods, a distinction that might have been difficult without culture methods. In studies from our laboratories [24], the use of native-state histoculture has been applied to distinguish small-cell from non-small-cell lung tumors. This study included 78 patients with non-small lung cancer and 13 with small-cell lung cancer with an over-all culture success rate of 92%. Tumor types exhibited their typical three-dimensional in vivo tissue architecture in vitro. In vitro proliferation rates were measured by histologic autoradiography after four-day incorporation of [3H]thymidine. The percentage of cells radiolabeled in the most proliferatively-active regions of the autoradiograms were termed the growth fraction index. The growth fraction index for pure small-cell lung cancer was 79 ± 10% differing markedly from that of 35 ± 19% for mixed small-cell/large-cell tumors; adenocarcinoma 38 ± 16%; large-cell undifferentiated carcinoma (40 ± 18%) and squamous cell carcinoma (33 ± 15%). It was concluded that the three-dimensional sponge-matrix histoculture system is a useful means of distinguishing pure small-cell lung carcinoma from non-small-cell lung carcinoma which may improve diagnosis and, thereby, treatment decision-making.

The possibility to identify tumorspecific new drugs in native-state histoculture

The key aspect in the development of new drugs is to determine if they are tumor specific. Many drugs are cytotoxic but most, if not all, in our current armamentarium seem to lack tumor specificity. One approach to this problem is to measure the comparative response of normal and malignant tissue in culture to drugs. This idea had its genesis a long time ago. For example, Cormann [48] used a row of tumor explants and a row of normal tissue explants placed in the same tube with drugs added to the medium. The comparative effects on the tumor and normal were evaluated. We have done similar comparative drug-response experiments using the native-state sponge-matrix histoculture system to culture normal and tumor tissue from the same breast on parallel gels for example. As can be seen from Fig. 3, there is no tumor selec-
Fig. 3. Comparison of drug response of human normal versus human malignant breast tissue in three-dimensional native-state sponge-gel-matrix histoculture.

Tissues were cultured, incubated with [3H]thymidine and prepared for autoradiography as described in Fig. 1. The slides were then analyzed in a blinded fashion with a microscope epi-illuminated with light polarized by an IGS cube (Nikon). Replicating cells were determined by the presence of bright green-reflecting silver grains over the cell nuclei that had incorporated [3H]TdR. Cells were considered labeled if one- or more grains were present over the cell nucleus. Benign stromal cells were excluded from the measurement by their morphological appearance. When counting, each slide was scanned at X 40 or X 100 power to locate the areas of maximum label. Once these areas were ascertained, the number of cells undergoing DNA synthesis was determined in at least 3 of the visual fields for each tumor piece at X 200. The growth-fraction index was calculated by dividing the number of labeled tumor cells by the number of total tumor cells. Drug-treated cultures were compared to untreated controls.

Concentrations of drugs used include doxorubicin (Adria) 29 ng/ml; melphanal (Melph) 1.0 μg/ml; methotrexate (MTX) 2.25 μg/ml; 5-fluourouracil (5 FU) 4 μg/ml; vincristine 23 ng/ml. These concentrations are referred to in the manuscript as the X-concentrations which correspond to clinical achievable doses in vivo. Also used in this study were 10X drug concentrations. Exposure times for all drugs were 24 hours in vitro.
tivity of the standard chemotherapy drugs tested. Indeed, the tumor in this case is more resistant than the normal tissue. However, this comparative approach allows one to identify tumor-specific drugs in a screen or an evaluation program.

Clinical trials
The value of any clinically-related evaluation system must be reflected in good correlation between the measurement made in vitro and in vivo. As seen above, using the model system of human tumors growing in nude mice and in vitro in native-state sponge-matrix histoculture, there is a reasonably strong correlation between in vitro and in vivo response to drugs [26]. Prospective clinical trials are really the only rigorous means of determining clinical correlations. In this regard a study of head and neck cancer is being carried out by our group evaluating the effect of cisplatin, 5-fluorouracil and the combined effects of 5-fluorouracil and cisplatin in native-state sponge-matrix histoculture and in the patient. Results obtained thus far have indicated 100% correlation between in vitro drug response and clinical outcome in a small set of patients [49]. Further trials are in process. Leighton [28] has stated that the tumor in vitro and in vivo must be in a similar physiological state in order to get good in vitro/in vivo drug-response correlations; in other words, the tumor in vitro cannot be dying or maturing while in vivo it is rapidly proliferating. Secondly, as mentioned above, the choice of the proper endpoint will be critical in determining accurate prediction of sensitivity in vivo.

Possibilities

Individual drug-response assay available to large numbers of cancer patients at low cost for all tumor types
The development of an individualized drug-response assay for all cancer patients undergoing surgery or biopsy available on a mass scale at low cost for all tumor types with a high rate of evaluability and high rate of accuracy of prediction is possible using the native-state sponge-matrix histoculture system. The key elements will be the demonstration of in vitro/patient drug-response correlation and reducing the labor so that costs can be lowered. Lowering of costs will largely depend on using easily-measured endpoints of drug response such as scintillation counting of [3H]-thymidine uptake. Experiments are currently in progress to determine if this can be the case.

Phillips et al [54] questions the value of in vitro sensitivity assays, especially for identifying effective cancer chemotherapeutic agents. In particular they cite potential problems concerning the biology of solid tumors in vivo including drug penetration barriers, proliferation gradients and microenvironmental conditions. Phillips et al suggest that in light of the above parameters, it may be more useful to study three-dimensional tissue in vitro rather than clones or monolayers. However, even with the use of three-dimensional tumor tissue in vitro, the above potential problems must be taken into account.

Effective drug evaluation/screening
It is critical to be able to reduce the procedures involved in the native-state sponge-matrix histoculture drug-response system so that large-scale screening can take place. It is also important to compare drug response in normal tissues vs. tumor tissue. Normal tissues should include normal tissue corresponding to the tumor and also very drug-sensitive normal tissues such as bone marrow and intestinal mucosa. The idea will be to miniaturize the histoculture system into 96-well dishes, with the use of the endpoint of scintillation counting of [3H]-thymidine incorporated into the DNA of tissue in each well or with the use of colorometric reactions such as with fluorescent vital dyes as endpoints.

Evaluation of immunotoxins and other immunological effectors in the native-state system
The evaluation of efficacy of immune-system-based therapy will depend on the co-incubation of the cultured tumors in the native-state sponge-matrix histoculture system with immunotoxins or activated immune cells such as lymphokine activated killer cells (LAK) which may be dependent on specific antibody reactions with the
histocultured tumors (antibody-dependent cellular cytotoxicity). Since three-dimensional tissue is used, studies of immune-cell or immunotoxin accessibility into the tumor tissue can also be measured as well as efficacy.

The evaluation of drug response of cells of high metastatic potential in the native-state system

Leighton [28] has described key studies of in vitro tumor invasion using sponge-matrix cultures. The particular aspect of the metastatic process measured by Leighton was the interaction between tumor cells and stromal cells which may be the essence of metastasis. The native-state sponge-matrix histoculture system thus offers the possibilities of determining if the cells identified as being metastatic by their invasive properties into the gel matrix can be targeted by specific drugs.

The possibility of studying tumor-specific nutritional requirements in the native-state system to selectively starve tumors and enhance their chemosensitivity

Another key aspect now possible with the native-state sponge-matrix histoculture system is to be able to study the specific nutritional requirements of tumor tissue in comparison with normal tissue. For example, in monolayer culture, cell lines derived from a large percentage of tumors have an absolute dependence on preformed methionine in the culture medium, whereas normal cell types can survive and grow in the presence of homocysteine in place of methionine in the culture medium used [50, 51]. Nutritional-requirement studies need to be made on surgical specimens in native-state sponge-matrix histoculture of tumor and normal tissue with the goal of trying to be able to selectively starve tumors by depriving the tumors of tumor-specific nutritional requirements. Methionine-dependent tumor cells, starved for methionine in vitro, become selectively synchronously arrested and thereby much more susceptible to cell cycle-specific drugs [52]. It is important to apply this approach in vivo.

The establishment of permanent histolines in the native-state system

As described above, Slocum et al [47] have been able to serially passage surgical specimens from one gel to another in the native-state sponge-matrix histoculture system. This can be done on a wide range of tumor types. There will be, then, a source of passagable tumor tissue in vitro to study various aspects of the mechanism of carcinogenesis or its reversal as well as to study new treatment strategies.

Acknowledgement. The manuscript was expertly word-processed by Polly Jayne Pomeroy, whom we greatly appreciate.