In Vitro Drug Response Assays for Entry into the Rational Era Cancer Chemotherapy

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I. THE PROBLEM

"Histologically identical tumors often differ in their response to treatment" -Black and Speer, 1954.

Human tumors are complex systems often consisting of multiple classes of cancer cells, in addition to supporting stroma, normal cells, and lymphocytes. Each tumor is a unique, dynamic system. Even tumors that are classified under the same histotype are highly individualistic. Researchers have known this characteristic for a long time and have concluded that each tumor has a specific drug-response spectrum. The major problem has been how to devise a system in which to accurately predict the drug-response of individual patients' tumors. Both the method of culturing the tumors and the end-point to be measured as response to drug treatment are of critical importance. There have been many early attempts to design in vitro systems to culture tumor cells and tissues(1-16). Among the earliest was the determination of drug sensitivity in tumor slices by Black and Speer in the early 1950's (16) using tetrazolium-dye reduction as an endpoint. The work of Black and Speer was ahead of its time and of fundamental importance. In this review, the major attempts to design clinically-useful in vitro drug response assays will be critically considered.

II. CLONOGENIC ASSAYS, ORIGINS AND POSITIVE POINTS

A number of researchers attempted to grow myeloma and various other types of tumor cells in agar based on observations of Puck and Marcus(17) that normal fibroblasts would not grow in agar, and tumor cells would. Therefore, potentially selective systems were devised for disaggregated tumor cells to grow in the absence of an overgrowth of fibroblasts. The first attempts at agar assay of primary tumor cells were done with mouse myeloma (Park et al. [18]; Ogawa et al.[19]). Subsequently human myeloma (Hamburger and Salmon [20]) as well as other types of cancer (Hamburger and Salmon [20]; Courteney and Mills [21]; Courteney et al. [22]; Salmon et al. [23]) were cultured in agar and tested for drug response.

As Weisenthal and Lippman (24) point out, clonogenic assays were considered to be the "gold standard assay" in radiobiology by the time of the 1970s. Roper and Drewinko(25) demonstrated that clonogenic assays
in established cell lines differed in results from non-clonogenic assays. Steele(26) stated that cells which could form clones in agar were the stem cells of tumor. Salmon et al.(23) then reported positive correlations between in vitro response of tumor cells in soft agar and clinical response. As Weisenthal and Lippman(24) stated, the latter three publications stimulated a world-wide interest in using clonogenic assays for clinical drug testing and pre-clinical drug screening(18, 20 - 22, 26 - 124).

Von Hoff (125) has recently reviewed these studies on clonogenic assays. Over 40 types of cancer have been culturea in the agar colony formation assay and evaluated for drug response. Over 14,000 specimens have been cultured in agar of which close to 5,000 have been evaluated for drug response. Von Hoff (125) has reviewed 54 different in vitro-in vivo clinical correlation trial from 35 institutions performed over the past 12 years. The combination of the clinical trials has resulted in 2,300 correlations. The sensitivity of the combined clonogenic assays [true positive / (true positives+false negatives)] is 79%. Specificity of the test [true negatives/ (true negatives+false positives)] is 86%. The predictive value negative [true negatives/ (true negatives + false negatives)] is 91%. Von Hoff (125) points out the results are as impressive as the results reported for the estrogen receptor assay or for in vitro antibiotic sensitivity testing. In initial studies comparing clinical outcome when patients are treated with results of the assay vs. clinician choice there seemed to be greater responses among patients treated by the results of the assay (126). In related trials, of 21 patients with gastro-intestinal cancers, 3 out of 11 patients treated with drugs to which their tumors were insensitive died within two years of surgery, in comparison to the survival of 10 out of 10 patients for at least 4 years treated with drugs to which their tumors were sensitive in the clonogenic assay (187). That sensitivity assays may increase patient response and survival is a very important point (126 - 127). However, the number of evaluable patients is small in these efficacy studies. In addition, clonogenic assays have correlated well with assays in nude mice carrying the same human tumors assayed in vitro, which indicates clonogenic assays could, at least in some cases, re-

place animal studies for cancer drug evaluation (184 - 186).

III. PROBLEMS WITH CLONOGENIC ASSAYS

However, the clonogenic assay technique has drawbacks. It was found that a very low percentage of disaggregated tumor cells, in the order of 1 in 10⁴ or lower, would grow in agar-based medium. The cells that did grow were termed tumor stem cells (Salmon et al. (23)), as indicated above, although it was never made clear what the relationship was of cells which would grow in agar to particular malignant cell types. As Von Hoff (125) points out, in his laboratory in San Antonio, of approximately 14,000 patient tumors attempted to be grown, only 30% of the cases have yielded evaluable results. In addition, the assay takes a number of weeks to yield useful data. Second-generation techniques modifying the clonogenic assay, for example, using rat erythrocytes, low O₂ atmosphere and tubes (21, 22), capillary cloning (109, 123), and [³H] thymidine incorporation (128, 129), have helped to increase the number of specimens that can be evaluated for drug sensitivity. Von Hoff (125) points out that growth is best in the clonogenic assay for cancers of the ovary, uterus, kidney, brain, and mesothelioma.

Cloning assays also have the problem of the use of multiple drugs in the testing process since it is difficult to add drugs sequentially to the culture system. Quality control has also been a problem with the cloning assays, in particular difficulties of distinguishing clones from cell clumps. However, these problems seem to have been somewhat reduced (125). Weisenthal and Lippman (24) argue, however, that aggregates which seem to be colonies pose a large problem in the clonogenic assay, although Von Hoff (125) states that this is no longer a problem. Weisenthal and Lippman (24) state that the clumps may create a bias in assays that predict drug inactivity in that the clumping could produce an artificial but coincidentally accurate true-negative rate in clinical correlation studies in which most patients have tumors that are chemo-resistant. In this light, Weisenthal et al.(130) noted that in the clonogenic assays, 60 - 65% of specimens were not sensitive to spirogermanium, sangivamycin, and homoharringtonine. Other assays have shown essentially
all types, normal and neoplastic, are sensitive to these 
drugs, indicating that the clonogenic assays results 
with these agents were really false negatives, and 
according to the authors, probably due to clumping 
artifacts.

Weisenthal and Lippman (24) note, with regard to 
theoretical problems of the clonogenic assay, that cell 
disaggregation necessary for plating in the clonogenic 
assay results in a loss of normal cell interactions and 
three-dimensionality which may be critical in drug 
response (131, 132). Spheroids (133 - 135) can be con-
structed from disaggregated cells to form three-
dimensional structures. Similar to solid tumors, the 
interior may be hypoxic and at low pH, and interior 
cells may not be accessible to metabolites and drugs 
as the exterior cells. Of critical importance, when drug 
response was compared by Bhuyn’s group (135) in 
spheroids to single-cell suspension, the spheroids were 
much more resistant to antimetabolites such as cyto-
sine arabinoside and methotrexate, >11-fold and> 
125-fold, respectively, and somewhat more resistant to 
vincristine, doxorubicin, and other antibiotics. Alky-
lating agents affected both cellular configurations 
equally. These results emphasize the importance of 
three-dimensionality. Indeed, in recent experiments, 
Bhuyn’s group (136) has seen for certain drugs such as 
Pierrindin C that colon carcinoma cells are sensitive as 
single cell structures but are importantly, resistant as 
three-dimensional slices in vitro. These results empha-
size the importance of three-dimensional intact-tissue 
culture (see below).

Reversibly non-dividing (G0) cells are probably not 
assayed in clonogenic assays. Cells assayed are ac-
tively dividing and may be the most sensitive of the 
tumor cells to chemotherapy. Actual stem cells of the 
tumor that showed the capability for potential contin-
ual self-renewal in vitro could be mainly G0 cells in 
vitro and may not be assayed because of their inability 
to be directly cultured in clonogenic assay. On the 
other hand, tumors cells committed to differentiation 
and thereby of lesser malignancy may selectively 
develop colonies in vitro (Weisenthal and Lippman 
[24]).

IV. CLONOGENIC-LIKE ASSAYS

Kern and Weisenthal (137) have significantly modi-
fied the clonogenic assay. The main modifications are, 
in addition to measuring colony formation, measuring 
[3H] thymidine incorporation in the cells incubated in 
the agar-based medium. In addition, Kern and Weisen-
thal used products of drug concentration x time expo-
sure that were approximately 100-fold higher than 
those achievable clinically. They found for patients 
whose tumors showed a less-than-40% thymidine-
incorporation inhibition or less-than-15% colony-
formation that the assay is 99.2% specific and 43% 
sensitive in identifying the patients as drug-resistant. 
Only one of the 127 patients with tumors showing 
“extreme drug resistance,” as these authors termed it, 
responded to chemotherapy. Kern and Weisenthal 
state a high specificity for drug resistance was possible 
because the drugs were tested at very high concen-
tration x time exposures in vitro.

18 - 42% of the patients, depending on the tumor 
types, had specimens which showed this extreme drug 
resistance with essentially none of the patients re-
sponding to chemotherapy (Kern and Weisenthal[137]). 
This broad resistance was seen over a wide range of 
cancer types including those that were otherwise 
usually relatively high responders (Kern and Weisen-
thal[137]).

Kern and Weisenthal (137) point out the applications 
of their highly-specific drug-resistance assay include 
avoiding the use of regimens on patients containing 
inactive agents, in particular the individual agents for 
breast cancer, including CMF (cyclophosphamide, 
methotrexate, and 5FU), CAF (cyclophosphamide, 
doxorubicin, and 5FU) or CA (cyclophosphamide, 
doxorubicin). Kern and Weisenthal’s assay (137) can 
also reduce treatment-induced morbidity in patients 
with highly drug-resistant tumors and can identify 
patients with highly resistant tumors as candidates for 
experimental therapy such as bone marrow transplant 
and other experimental therapies. The Kern-
Weisenthal assay (137) however suffers significantly 
due to its inability to accurately identify drug-sensitive 
tumors.

Another modification of the clonogenic assay, the
disc-diffusion, soft-agar, colony-formation assay, has been developed by Corbett and colleagues (138) and modified by Smith et al. (139) to screen drugs which are claimed to be selective against solid tumors. In this assay selectivity is evaluated by co-culturing the L 1.210 leukemia line, which is sensitive to most currently used drugs, and a drug-insensitive solid tumor such as pancreatic or colon adenocarcinoma. The colonies of the two cancer types are morphologically distinct. The test agent is placed on top of the agar previously seeded with the two cancer types. The authors have found that if an agent has cellular selectivity for a solid tumor as opposed to leukemia selectivity in the disc-diffusion assay it is more likely to be active against different types of solid tumors in vivo. The assay needs clinical validation. On a theoretical basis, it is not clear what, indeed, generally distinguishes solid tumor cells and leukemia cells growing in the highly selective artificial agar-based growth system where growth kinetics of both cell types could be similar.

Another modification of the clonogenic assay is to culture cells on an adhesive matrix (140). However, there is a question of what cell types adhere to the matrix, in particular whether fibroblasts are predominant. Another variation of the clonogenic assay used a medium developed specifically for mammary epithelium and irradiated fibroblasts as a substrate. This assay was able to accurately predict drug sensitivity as well as drug-resistance in breast cancer (144). If the high accuracy can be repeated independently, this assay could be used more extensively in breast cancer. A similar approach has been taken in colon carcinoma which also seems promising. (142)

V. CELL-TOXICITY END-POINT ASSAYS

Pavlic et al. (143) point out that the standard clonogenic assay, although showing promise and identifying drug resistance as stated above, has been only moderately successful in predicting clinical sensitivity, 40–70% depending on the laboratory. Pavlic et al. (143) point out that one explanation for the failure of predicting sensitivity is that the clonogenic assay does not provide an estimate for potential recovery from the cytostatic effects of drugs which can lead to false-positive results. The clonogenic assay does not distinguish cell kill from a temporary halt in cellular reproduction, leaving cells viable.

The 1–2 log cell kill that is able to be measured in clonogenic assays may be insufficiently sensitive to predict in vivo drug sensitivity. In clonogenic assays sub-clinical concentrations may have to be used to avoid false positives. However, the sub-clinical low levels of drugs may preclude normal uptake by the cells of the drugs (24).

Weisenthal and Lippman (24) point out the time after drug exposure when end-points are measured is critical. These authors report that for HL60 promyelocytic leukemia cells viability decreased from 38% to 17% and 0% when assayed 2, 4 and 6 days, respectively, after 1-hour exposure to doxorubicin by means of a dye-exclusion assay. They emphasize the importance of waiting a sufficient time to make measurements following a cytotoxic insult.

The clonogenic assay does not determine tumor kill as mentioned above. In the studies of Pavlic et al. (143) viable cells were determined by the activity of intracellular esterases which convert a colorless fluorescein diacetate to green fluorescein. Non-viable cells are identified by their ability to take up propidium iodide which is taken up only by cells that have a non-intact cell membrane, binds to DNA and fluoresces red. Pavlic et al. (45) demonstrate that the exposure of tumor cells to even very high concentration of an anticancer agent can inhibit in vitro proliferation while esterase activity and the ability to exclude propidium iodide are persistently retained allowing the potential of recovery from drug exposure. However, when the viability of established cell lines used by Pavlic et al. (143) progressively declined on days 4 and 7 following exposure, and recovery did not occur. When proliferative recoveries did occur, the viabilities remained elevated on days 4 and 7.

Pavlic et al. (143) found that in vitro drug sensitivity based on proliferation criteria was associated with high-viability estimates in 22% of determinations that they performed on primary tumor cell preparations. Pavlic et al. (143) argued that the cell’s potential recovery may explain the excessive false positives in chemosensitivity assays based on proliferation end-
points.

In this light, dye exclusion as an end-point to measure cell viability or cell death allows one to measure the drug resistance of \( G_0 \) as well as proliferating cells. As Weisenthal and Lippman point out (24), the key critical technical problem in using dye exclusion as an end-point is that sufficient time must elapse following drug treatment for lethally damaged cells to lose their membrane integrity. This would, of course, depend upon the target of the chemotherapeutic drug. It is important to note that Weisenthal and Lippman (24) state that dye exclusion assays may be valuable in testing of neoplasms which proliferate poorly in culture. The assay developed by Weisenthal et al. (144) used disaggregated cells and Fast Green as their reporting dye which is excluded from living cells. Clinical correlations with dye exclusion as an end-point compare favorably with clonogenic assay. However, the Weisenthal assay may be better suited to hematological tumors than to solid malignancies since it is based on measurements on cells in suspension.

The Weisenthal assay has been used by Gazdar et al. (127) to measure drug sensitivity in small-cell lung cancer. Only about 25% of patients entered in the protocols could have a determination done, and the time to grow sufficient cells for assay (median 37 days) was only sufficient secondary treatment. However, there was a correlation between \textit{in vitro} sensitivity and \textit{in vivo} response. In addition, patients whose tumors had \textit{in vitro} responses had a tendency to longer survival (127). It seems, however, for small-cell lung cancers, more efficient and rapid \textit{in vitro} systems are necessary. Weisenthal et al. (198) found for non-small cell lung cancer their assay could classify patients into low- and high-probability responders and average-and below average-durations of survival.

VI. ASSAYS USING THREE-DIMENSIONAL TISSUES IN VITRO

Initially, investigators attributed the drug resistance of spheroids to poor diffusion of the drugs to interior cells of the structures and the relatively high proportion of resting cells in spheroids, which could be a result of nutrient deprivation and hypoxia. However, Heppner and her co-workers performed experiments that demonstrated that three-dimensional structure itself accounts for drug resistance rather than just simple inaccessibility to nutrients (132, 189, 150). Heppner and colleagues embedded cell clusters or fragments in collagen gels, exposed the culture to various cytotoxic drugs and compared the drug responses of the same cells in monolayers. They found that the increased resistance in the three-dimensional cultures could be up to 1000-fold greater than in monolayer cultures, for example, with melphalan. In three-dimensional bolus cultures in collagen, the cells grew even in the presence of drug concentrations that reduced survival in monolayers to less than 0.1% of controls. Heppner and colleagues observed that if the cells from the collagen were replated as monolayers they became sensitive again. The key experiment done by Heppner and colleagues which demonstrated that the tissue architecture itself is critical in determining sensitivity was that when monolayer cultures were treated with melphalan and 5-fluorouracil and subsequently transferred to collagen gels, the cells became highly resistant to these drugs. Thus, the cells were exposed to the drugs as monolayers where the drugs can access the cells readily and diffusion is not limiting. However, once the cells were transferred after drug exposure to a three-dimensional structure, the structure seemed to confer high resistance to the drugs.

In this light, an illustrative set of experiments is described comparing drug resistance in tumors grown in animals to the drug resistance of the tumors in monolayer vs. three-dimensional culture. Teicher, et al. (191) serially passaged the EMT-6 tumor in mice that were treated with cisplatin, cyclophosphamide and thiotepa over a 6-month period with a total of 10 passages. This treatment induced highly-resistant tumors \textit{in vivo}. However, when these tumors were grown as monolayer cultures, they were as drug-sensitive as the parental cells. It was concluded by the authors that certain types of drug sensitivity may only be expressed \textit{in vivo}. However, when Kerble et al. (197) grew these same \textit{in vivo} drug-resistant tumor cell lines as spheroids in three-dimensional cultures, resistance was observed up to almost 5000 times that of the parent with certain drugs, for example, 4-hydroperox-
cyclophosphamide (4-HO₂-CTX), the active form of cyclophosphamide used in vitro. High resistance was also observed to cisplatin and thiotepa. This resistance was not observed in monolayer culture, even when the monolayers were cultured on laminin or matrigel. Kerbel and colleagues (197) observed that while the parental non-resistant tumor lines formed spheroids that were loose and grape-like multicellular aggregates that were ellipsoidal in shape, every resistant tumor sub-line formed a very highly compact spheroid. It should also be noted that in vitro exposure of the parental EMT-6 cells to 4-HO₂-CTX induced the formation of compact spheroids just as the in vivo exposure did as described above. Thus, the experiments by Kerbel reconfirm that cells in a three-dimensional configuration can be much more resistant than monolayers of the same cells in vitro, and for the first time demonstrate that three-dimensional cellular configurations may become resistant to supra- pharmacological doses of drugs by forming compact structures. The compact nature of the spheroids may confer a "tissue-based" resistance to drugs (197) as opposed to cellular resistance seen only in vitro indicated by membrane pumps which reduce drug concentration (205).

It should be noted that Leighton, in the early 1950’s (199), observed that tumors grew as aggregates in histocultures and suggested that these aggregates were the units of metastasis rather than single cells by observing their migration in sponge-gel matrices. In this light, Kerbel et al. (197) have observed that the EMT-6 cells selected to be highly resistant in vitro are highly metastatic. These results have deep implications in that, indeed, if drug resistance generated in vitro is mediated by formation of tight aggregates, drug resistance may promote metastasis. Thus, the generation of drug resistance could greatly enhance the malignancy of tumors. Therefor, failure of drug treatment of patients may have a double ramification: not only will the tumor still remain viable despite drug treatment, but, indeed, the tumor could become more highly malignant because of the formation of highly aggregated emboli.

Greater resistance in three-dimensional vs. two-dimensional culture extends also to radiation. Olive and Durand (192) observed that Chinese hamster V79 cells grown as spheroids in suspension culture are more resistant to killing by ionizing radiation than are the same cells grown as monolayers. They found that the rate of DNA repair to radiation damage was faster in the DNA of cells in spheroids than in monolayers. Differential responses were found to be lost 8 hours after separation of the spheroids into individual cells which correspond to their loss of radioresistance. They also observed that spheroid cells showed fewer numbers of induced mutations per unit of radiation. Possibly DNA repair is also enhanced after chemotherapeutic-induced damage to the DNA in spheroids, or other three-dimensional configurations compared to monolayers, thereby possibly contributing to the enhanced drug resistance frequently seen in three-dimensional cultures.

Furukawa et al. (200) have cultured human tumors in three-dimensional collagen sponge-gel matrix culture (see below) with an endpoint of 3- (4,5-dimethylthiazol - 2- yl) - 2,5 - diphenyl - 2 tetrazolium bromide (MTT) reduction. The three-dimensionally growing cells on the collagen gel matrix were 2-to 180-fold more resistant to mitomycin C, doxorubicin, 5-fluorouracil, and cisplatin than those cells in monolayer culture. Furthermore, the influence of fibroblasts in the collagen gel matrix on the chemosensitivity of cancer cells was less than that in monolayer culture. These results confirmed for intact tissue what was observed in spheroids and cell boluses with regard to the effect of 3-dimensionality on drug response.

With regard to the advantages of measuring cell viability by dye exclusion and also preserving 3-dimensional cell-cell interactions, Rotman et al. (145 - 147) propose an approach to in vitro chemotherapeutic assays in a procedure they term fluorescent cytoprinting. Specimens in this procedure are mechanically or enzymatically mildly digested into cellular clusters termed micro-organs which the authors claim are exclusively tumor cells. The micro-organs are exposed to fluorescein acetate. Those that are fluorescent are viable and are collected with a Pasteur pipette and explanted on a support of cellulose fibers impregnated with collagen. The drug toxicity is measured by comparing the fluorescence as seen microscopically before and after drug treatment. As the authors point
out a key feature of fluorescent cytoprinting is that the cytotoxic effects are not measured against control cultures but against the base-line provided by the cytoprint of the same culture before drug addition. A high rate of evalubility has been demonstrated by these authors and high in vitro-in vivo correlations have been reported (Rotman et al. [145–147]).

The potential usefulness of the fluorescent cytoprint assay (FCA) was assessed retrospectively in 73 cancer patients by correlating individual tumor chemosensitivity in vitro with patient responses to chemotherapy by Leone et al. (192). The data show that FCA has a specificity of 98%, sensitivity of 81%, and predictive accuracies of 85% and 97% for positive and negative clinical responses, respectively.

Short-term assays based on incorporation of nucleic acid precursors into tumor fragments have been utilized in some clinical studies (148,149). The studies indicate a high correlation of 92% between in vitro clinical sensitivity and in vivo response with tumor metastases but with primary tumors only about 62%. These assays can have a high rate of false negatives. The methodology involves cutting the tumors into 1mm³ fragments for incubation for 3 hours with drugs. During the last hour of incubation the tumor pieces are exposed to [³H]thymidine and [³H]uridine followed by a chase of labeled precursors from intracellular spaces at 4°C with a 100-fold concentrated solution of unlabeled precursors. As Weisenthal and Lippman (24) point out, these studies are subject to numerous pitfalls relating to artifactual alterations in intracellular pool sizes and salvage vs. de novo deoxythymidine monophosphate synthesis. As Weisenthal and Lippman (24) state, if labeling could be delayed for a number of generations after drug exposure, the artifacts would be less pronounced and more accurate estimates of the number of surviving proliferating cells could be obtained. On the other hand, the relatively non-physiological conditions used in this system would probably not support the long-term culture needed for delayed assay. However, recent studies of the short-term assay for germ-cell tumors indicated not only in vitro-clinical correlations (90% true-negative rate, 54% true-positive rate) but longer survival for the patients with tumors sensitive to drugs in vitro (148).

VII. THE NEW GENERATION OF RATIONAL CHEMOTHERAPY BASED ON CLINIVALLY RELEVANT IN VITRO ASSAYS

The foregoing, however, make it obvious that a next-generation of technology is necessary for in vitro drug response assessment. It would be imperative in the next generation that the technology allow the development of a in vitro model that more closely resembles the in vivo situation. Leighton (150) in 1951 brought tissue culture closer to the in vivo situation by introducing a three-dimensional sponge matrix system. Leighton's system employed a cellulose sponge matrix that was impregnated with a plasma clot. The system was later modified by Leighton (151) by using collagen-coated cellulose-coated sponges which would be suitable to measure growing carcinomas, many of which were able to lyse the clot. Leighton was able to grow the Walker carcinoma as well as several other tumors such as mouse mammary carcinomas and rat hepatomas and embryonic tissues with maintenance of in vivo-like architecture. It was reported that the growth characteristics of the tumor and embryonic tissues were better in the collagen-coated cellulose sponges compared to either collagen sponges or collagen sponges alone. Leighton (201) has since taken three dimensional sponge-gel histoculture to a higher level in which he calls histologic gradient culture. This type of culture is carried out in chambers such that metabolic exchange between the pool of medium and the culture chamber takes place by diffusion across a collagen membrane roof of the chamber, thereby mimicking diffusion in tissues. Very realistic tissue formation takes place in such gradient cultures.

Sherwin and Richters (152) have termed the approach of maintaining in vivo-like tissue architecture in vitro "histoculture." Their approach was to explant 2-mm³ tissues about 1-mm thick, which would allow for optimal diffusion, which is very important as pointed out in the original tissue culture article by Alexis Carrel (153).

These ideas were further developed by Acedia et al. (154–155) who cultured embryonic lung tissue on sponges derived from pigskin. This approach was also further developed by Douglas et al. (156).
Three-dimensional culture of organ was initially advanced by Browning and Trier (157) and by Schiff (158). Their method kept fragments of human small intestine in contact but above the culture medium by explanting the tissue on a matrix partially above the medium level, or by rocking the culture, which alternated the position of the tissue in and out of the medium. Autrup (159) used a 95% O₂ / 5% CO₂ mixture as an atmosphere to enhance survival. Autrup has extended the growth capability of colon extensively by using gelatin sponges as a growth support and the rocking method alluded to above allowing the maintenance of normal colonic mucosa for at least 28 days. Great care was taken to avoid ischemia. The mucosa layers of colon specimens are dissected away from the muscularis externa and put on the sponges to maximize the diffusion of nutrients and oxygen into the tissue by stretching the tissue in the sponges (Autrup [160]).

Hoffman et al. (161–165, 202) have utilized the gel-supported histoculture approach to develop a next generation of in vitro chemotherapeutic assays. The system allows for the culturing of over 20 tumor types at an 90% or better evalability. The main advantages of the histoculture drug response assay (HDRA) are:

a) Cultures tissue in three dimensions, preserving native tissue architecture
b) Can use morphological end-points
c) 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
d) 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 (194)
e) Metabolic activity of the tumors can be measured by tetrazolium dye reduction biochemically (200), as well as by image analysis with concomitant observation of histology (203, 204)
f) Has a very high rate of evaluation for all tumor types—approximately 90% or better

g) Multiple agents can be tested sequentially
h) Uses relatively small amounts of tissue as small as 100mg
i) Has very low frequency of false negatives and false positives (202)
j) Normal as well as tumor tissue can be cultured in parallel to assess the tumor specificity of anti-neoplastic agents
k) Cytotoxic as well as biological drugs and activated immune cells can be assayed
l) Depending on end-point chosen, data can be obtained in 3–14 days
m) Correlates with patient drug response and survival

Vescio et al. (1991), in a preclinical in vitro-in vivo correlative study of the HDRA, reported a relatively high in vitro-in vivo correlation of drug response using human tumor lines in the HDRA and in nude mice. Histological autoradiography of ³H-TdR incorporation was used as the endpoint. The correlation rate varied depending on the drug used, however.

In a second preclinical in vitro-in vivo correlative study of the HDRA, the MTT end point was applied to histocultures in an attempt to increase in vitro-in vivo correlation (202). The chemosensitivities of 16 human tumor lines were determined in vitro by the HDRA, and retrospectively correlated to their in vivo chemosensitivity as xenografts in nude mice. The in vitro test was considered to be positive if tumor cell MTT reduction activity was lowered by more than 50%. The overall correlation rate of the efficacy results of the drug response assay to in vivo chemosensitivities was 89.8%, with 90.0% true-positive and 89.7% true-negative rates, 81.7% sensitivity and 94.6% specificity, thereby, indicating potential clinical use for tumor histoculture with the MTT end point. The MTT endpoint in this assay appears to have a higher ratio of correlating with chemosensitivity in vivo than the [³H] TdR-incorporation endpoint.

In clinical trials to determine the validity of the HDRA, 38 patients with measurable gastrointestinal carcinoma lesions had their tumor tested in HDRA with mitomycin C, doxorubicin, 5-fluorouracil and cisplatin (206). Of the 29 patients whose tumors showed drug resistance in the HDRA, 29 failed treatment with
one or more of these agents. Of the nine patients whose tumors showed drug sensitivity in HDRA, six had chemoresponses with 2 complete responses (CR) and 4 partial responses (PR) for a total accuracy of 92.1% (35/38). Thirty-two patients with stage III and IV gastric cancer without remaining measurable lesions were treated adjuvantly with MMC and fluoropyrimidines. Of the 10 patients whose tumors were sensitive in HDRA, 9 were alive at 120 weeks. Of the 22 patients whose tumors were insensitive in HDRA, the 50% survival time was 49 weeks and only 6 were alive at 120 weeks. (p<0.01). Twenty-nine patients with stage III-V colorectal cancer without remaining measureable tumor lesions were treated adjuvantly with fluoropyrimidines. All the 7 patients whose tumors were sensitive to 5-FU in HDRA were alive at 120 weeks while 14 of 22 patients whose tumors were insensitive in HDRA were alive at 120 weeks. Thus the HDRA seems clinically useful in the treatment designs of gastrointestinal cancer.

The HDRA was recently evaluated in a blinded retrospective clinical trial and was found to correlate to drug sensitivity, resistance and patient survival. In order to further investigate the potential of HDRA to contribute to patient survival, 215 patients with gastric cancer from 45 medical centers were tested with the HDRA in a blinded study after resection of the primary lesion. One hundred and sixty-eight patients received at least 20 mg/m² of mitomycin C (MMC) and a minimum of 30 g/body of UFT, a mixture of tegafur and uracil at a molar ratio of 1:4 thereby making them eligible for the study. Of these cases, 128 were evaluable by the HDRA. The evaluable patient tumors were tested by the HDRA with the [3H] thymidine incorporation endpoint measured by microautoradiography to be drug sensitive or resistant. In in vitro conditions for distinguishing sensitivity and resistance that matched the response rates for historical controls for gastric carcinoma were 90% IR and 0.12 ug/ml for MMC and 70% IR and 1 ug/ml for 5-FU, respectively. Most importantly in the blinded study, the overall and disease-free survival rates of the HDRA Sensitive Group were found to be significantly higher than that of the HDRA Resistant Group tested under the above conditions. The data further indicate the importance of three-dimensional tumor culture for obtaining accurate clinical information. The results demonstrate that the HDRA response correlates to patient survival which suggests the potential of the HDRA to contribute to patient survival in gastric cancer when used prospectively (207).

To determine whether the HDRA assay correlates clinically in head and neck cancer, the in vivo / in vitro effects of cisplatin (DDP) were compared in 23 of 26 patients with head and neck cancer, which is an 88% evaluation rate (208). The criterion for in vitro sensitivity to cisplatin was an 84% or greater inhibition of the number of [3H] thymidine-incorporating cells compared to controls as measured by histological autoradiography. Comparisons were made to clinical response : (CR), (PR) ; or no response (NR). Ten of twelve patients with in vitro sensitive tumors had either CR or PR clinically. The overall accuracy of the HDRA was 74% in this correlative clinical trial. The predictive value positive was 83%, the sensitivity of the assay was 71%, and specificity 78%. Seven of eleven patients with in vitro resistant tumors demonstrated NR for a predictive value negative of 64%.

Recently the HDRA has been used for drug-response analyses of urological tumors with findings of a high culture success ratio, longevity in culture, maintenance of primary histopathology, and reproducible chemosensitivity response (166, 167, 181, 182).

In addition to being able to predict chemotherapeutic response, the degree of proliferation of cells within the histocultures correlates with the stage and grade of breast and ovarian tumors (164) and distinguishes small-cell from non-small-cell lung tumors (165).

VIII. PROGNOSIS OF SENSITIVITY ASSAYS

What does the future hold for in vitro sensitivity assays in cancer? The cumulative results from clonogenic systems, suspension cell systems, and three-dimensional systems argue that the concept is already providing clinical use. It is necessary to predict clinical sensitivity at greater accuracy in particular to identify effective antitumor agents.

With regard to new drug discovery, the monolayer human solid-tumor cell line drug-screening approach
of the National Cancer Institute (168–179) seems quite artifac-
tual in that monolayers are often more sensitive than three-
dimensional in vitro tissue or in vivo tissue (134–136) and the
cell lines are highly selected and may have little resemblance
to their original tumor. The most promising approach seems to
be the most in vivo-like three-dimensional histoculture
models along with multi-end-point analyses to select truly
tumor-specific drugs (76).

New subtle end-points such as those developed by Ogura et
al. (183) that measure drug response by intracellular mor-
phological changes should also help predict drug response.
Recently the endpoint of glucose consumption in three-
dimensional histoculture has shown that after many
weeks of histoculture tumors can recover from 5FU
treatments (195). Such as a repeatably measurable
non-invasive endpoint may provide critical data as to
what drugs may be able to induce durable responses.

Recently Kopf-Maier and Kolon (188) placed dense
cell suspensions of carcinomas on membrane filters at
the air-medium interface, resulting in growth of solid
nodules of organized carcinoma tissue. Determination of
viable cells was made by neutral red (NR) uptake
and local cell mass was measured by sulforhodamin B
(SRB). Determination of NR : SRB absorbance ratio,
related as percentage to control value indicated the
fraction of viable cells and gives a measure of the
cytotoxic injury caused by an applied cytotoxic drug.
Thus, this assay seems promising to determine sensi-
tivity and resistance of individual human carcinomas
in vitro using a 3-dimensional histoculture system with
relevant endpoints.

The most important current need in cancer therapy is
the development of cancer-specific drugs (Grindy [180])
which require relevant in vitro models that will ini-
tially identify them (Grindy [180]). With the discovery
of highly effective tumor-specific drugs, the true-
positive accuracy and usefulness of in vitro sensitivity
assays will rise.

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