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Perspectives and Commentaries

Clones, Dyes, Nuclides, Mouse Kidneys, and . . . Virions: A New-Clonogenic Assay for Tumor Chemosensitivity*

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(A COMMENT ON: Parsons PG, Maynard KR, Little JH, McLeod GR. Adenovirus replication as an *in vitro* probe for drug sensitivity in human tumors. *Eur J Cancer Clin Oncol* 1986, **22**, 401–409.)

THE WORDS in the title of this editorial refer to methods used for testing the chemosensitivity of fresh human tumor specimens. The existence of such a multitude of techniques implies that no one single technique is presently considered to be entirely satisfactory. This editorial will deal largely with the last of the above techniques, but I will begin with a brief consideration of some issues relating to all of the techniques.

The search for a useful tumor chemosensitivity test dates back at least to 1954 [1]. A large number of assay end points have been proposed [2–4]. During the last 10 years, clonogenic assays have been most widely studied. A variety of arguments have been advanced purporting to illustrate the superiority of this particular assay end point over the others [2, 3]. A particular argument commonly advanced has been the possibility that exposure to drugs or other lethal insults may produce a degree of cell damage which is not immediately apparent but which becomes evident only following the passage of multiple generations of cell divisions following the toxic insult. A counterargument is that a meaningful clinical response most probably requires the killing of multiple logs of proliferating cells [2]. The eventual killing of multiple logs of proliferating cells may, in turn, be predicted by the observation of early evidence of cell damage in the entire tumor cell population, as has been seen in many different *in vitro* and *in vivo* tumor systems [2]. A number of potentially useful assay systems are based upon the principle of measuring early tumor cell damage following toxic insults. These include assays based upon dye exclusion [5, 6], dye reduction [1, 7], incorporation of radioactive DNA precursors [8, 9], and measuring changes in the size of tumor fragments implanted underneath the renal capsule of normal mice [10]. A variety of additional arguments have also been advanced with regard to the validity of clonogenic vs. non-clonogenic end points [2].

Probably too much emphasis has been placed on the degree to which the physiology of a tumor assay system models the physiology of the tumor cells in the patient. It may be possible to obtain some very useful information from assay systems which are decidedly unphysiologic. Such an unphysiologic assay system is described by Parsons and co-workers in the current issue of this journal. For the purposes of a useful chemosensitivity assay, we must have a means of discriminating between cells which are sensitive to treatment with a given agent and cells which are resistant to treatment with the identical concentration or dose of the same agent. The mechanism of cytotoxicity induced by the drug may be entirely unrelated to what is being measured by the assay system and yet the assay system may accurately reflect the mechanism by which cells become resistant to the drug. As an example, consider the following situation.

There is considerable speculation that an important mechanism for resistance to certain drugs may be related to the ability of resistant cells to rid themselves of toxic agents through the use of an active membrane pump. A cell that was

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very efficient at pumping out unwanted substances could become resistant to a number of drugs with unrelated mechanisms of toxicity [11, 12]. Now, a certain concentration of drug might be required to interrupt long-term cell proliferation in 90% (one log) of cells, while a higher concentration of drug might be required to kill the same number of cells outright within the first cell cycle generation following drug exposure. Presumably, however, one could calibrate an assay system which measured long-term cell proliferation at a lower drug concentration than the drug concentration required to calibrate an assay system based upon early cell death. At the fixed drug concentrations thus calibrated for each assay system, both assay systems might be equally good at discriminating between populations of cells with differing abilities to pump out toxic substances. As a corollary to this, both assays might be equally accurate in detecting the activity of agent which could circumvent drug resistance by means of inactivating the membrane pump. If one were interested in identifying potential agents to be used for circumventing drug resistance through this mechanism, the choice of which assay to use would be based upon factors such as which systems were more practical and efficient.

The same sort of reasoning would also apply to the detection and circumvention of other mechanisms of resistance such as glutathione content and repair capacity of the tumor cells. If investigators were to put forth more effort into using presently available assay systems appropriately to discover and test new methods of circumventing drug resistance in fresh human tumor specimens, it is my prejudice that surprising progress in improving cancer chemotherapy might be achieved. It is time to stop quibbling about which approach to chemosensitivity-testing is most theoretically pure and intellectually satisfying, and to begin selecting assay systems for specific applications based upon their practicality and usefulness for these specific applications.

This brings us to a consideration of the potential practicality and usefulness of the adenovirus replication assay of Parsons *et al.*, described elsewhere in this issue.

These authors have described two separate assays, both based upon replication of adenovirus in the tumor cells to be measured. The first assay is termed the host cell reactivation assay (HCR). In this assay, the virus (a double-stranded DNA virus) is incubated with the drug to be tested and then the treated virus is added to the cultures of host tumor cells and allowed to replicate. At the conclusion of the assay, the cells are exposed to a virus antiserum and stained with immunoperoxidase.

The number of immunoperoxidase positive cells in the bottom of the microtiter wells are then counted and compared to control wells. Thus, the HCR assay compares the ability of host cells to repair the damaged viral DNA. This assay is obviously of use only in discriminating between cells in which the difference between sensitivity and resistance is based upon differing abilities of the cells to repair damaged DNA. The test might be expected to have some validity in measuring those treatment modalities dependent upon DNA damage, such as treatment with alkylating agents and radiation. One would not expect the test to be accurate in discriminating between sensitive and resistant cells in which resistance to an agent is related to factors such as the content of a target enzyme in the target cell, membrane transport of the antineoplastic drug, or glutathione content in the target cell. As a corollary, one would not expect the test to be useful for testing sensitizing agents which may act by means of inhibiting an enzyme pathway at an alternative site, inhibiting a membrane pump which rids the tumor cell of toxic drugs, or depleting intracellular glutathione. On the other hand, the test might well be useful in measuring the endogenous ability of different cells to repair DNA damage and to test possible sensitizing agents which may work by inhibiting these repair mechanisms.

A most exciting potential application of this technology is the ability to study DNA repair mechanisms in nondividing cells. For example, previous studies of radiation sensitivity have shown that proliferating cell populations are highly sensitive to radiation damage *in vitro*. Differences between the *in vitro* radiosensitivities of proliferating tumor cells are surprisingly small compared to the known differences between clinical radiosensitivities of different types of tumors. It is possible that clinical radiosensitivity may be more closely related to the ability of temporarily nondividing tumor cells to repair DNA damage, and the HCR assay offers a potential means of directly comparing the ability of nonproliferating, fresh human tumor specimens to repair DNA damage. Other potential advantages of this assay system include the requirement for relatively small numbers of tumor cells and the short time course required for the assay.

Experimental data suggesting the validity of the HCR assay include the following: first, there was a good correlation between the HCR assay and a cell survival assay based upon tritiated thymidine incorporation. These experiments were performed in established cell lines using MTIC, an agent thought to be cytotoxic by virtue of alkylating DNA. (Some reviewers might quarrel that the virus assays were not compared to clonogenic assays,

but tritiated thymidine incorporation assays have been shown by several authors to correlate closely with clonogenic assays—e.g. [9]). Additionally, the authors showed that the majority of primary cell cultures of malignant melanoma were "resistant" in the assay, which is what one would expect on the basis of clinical experience. Finally, there was a good correlation between the HCR assay in primary cell cultures from fresh human tumor specimens and the results in corresponding cell lines established from the primary tumors in three out of four cases.

A related, but potentially even more useful assay was termed the viral capacity assay. In this assay, the tumor cells (not the virus) were treated with drugs and the treated tumor cells were then infected with untreated virus. The same immunoperoxidase end point as in the HCR assay was utilized to determine how many cells were infected. The viral capacity assay is based upon the concept that some of the same key enzymes required for replication of host cell DNA may also be required for replication of viral DNA. If these enzymes are inhibited by antimetabolic drugs, then viral replication may be inhibited along with host cell replication. Examples of such key enzymes are: ribonucleotide reductase, dihyrofolate reductase, and thymidylate synthetase. It is also possible that any cytotoxic drug might produce a degree of nonspecific damage which would inhibit the ability of the cell to replicate viral DNA. Thus the viral capacity assay could potentially provide useful information about a wide spectrum of drugs including both specific antimetabolites as well as drugs which work through less specific mechanisms. There are some obvious theoretical problems, however. For example, some enzymes required to replicate viruses might not theoretically be identical to the corresponding enzymes required for cell division. Additionally, the quantity of these enzymes may be different in proliferating cells than in nonproliferating cells. If drug resistance were related to the quantity of a particular enzyme present within a proliferating cell, then performing tests in non-proliferating cells may or may not provide useful information about drug resistance.

The authors did compare the effect of two different antimetabolites (deoxyadenosine and hydroxyurea) in three different established cell lines. There was a good correlation between viral capacity assay and cell survival as determined by tritiated thymidine incorporation in two of three cell lines and only a moderate discrepancy in the third cell line. The authors then compared the activity of a large number oc cytotoxic agents on both cell survival and viral capacity in one of the established cell lines. In most cases, there was an excellent correlation between the cell survival and the viral capacity assay. The major discrepancy was in the case of melphalan, which was highly toxic to the cells but which had a negligible influence on viral capacity. It must be noted that these assays were performed on established cell lines in which there was presumably a very high percentage of proliferating cells. The authors did not test a viral capacity assay on fresh specimens of human tumor cells. Thus, we do not know whether or not viral capacity in non-proliferating cells will correlate with drug sensitivity and resistance *in vivo*. Despite the lack of correlative data in fresh human tumor specimens with the viral capacity assay, it is quite possible that this assay could be of value in testing for potential agents which may reverse drug resistance by means of mechanisms such as inhibiting the biosynthesis of a key molecule at an alternative enzyme site, inactivating a membrane pump for drug export, or depleting intracellular glutathione. The fact that this assay offers a potential means of studying such approaches to resistance modification in fresh human tumor specimens is an exciting possibility which should be explored.

A final note of caution, however, is that one must distinguish between effects on tumor cells vs. non-tumor cells in assays performed on fresh human tumor specimens. The authors claim that fibroblast contamination is not a problem under their growth conditions. Additionally, they state that the antiviral immunoperoxidase stain only labeled the nucleus of fibroblasts, while it labeled the entire cell in the case of tumor cells. Finally, they stated that macrophages washed away during their staining procedure, and thus there was no problem distinguishing between tumor cells and normal cells. It would appear that there might be a potential problem with tumors growing in suspension culture (such as hematologic neoplasms and small cell lung cancer), which may contain macrophages and other normal cells. Additionally, it is not certain that measures used to restrict fibroblast growth (such as reducing calcium in the culture media) would not also alter chemosensitivity in an artifactual manner. These and other issues must be addressed in future work.

The above reservations notwithstanding, it would appear that the creative technology presented here is likely to have a useful role for specific applications relating especially to (i) the study of drug and radiation resistance and (ii) the testing of methods to circumvent specific forms of drug and radiation resistance in primary cultures of human neoplasms. Its potential application as a clinical assay for use to "individualize" therapy is problematic, and requires much further work and clinical correlations. However, with the availability of this particular technology, as well as some other

types of technologies previously described [5–10], we may now have the tools to study drug resistance and its circumvention in fresh human tumor specimens. As more of this work is carried out in fresh human tumor specimens, I would predict that we will rapidly begin to see the identification of clinically-useful strategies to circumvent drug resistance.

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